MOLECULAR METHODS TO DETECT REGULATORY GENES IN ANAEROBIC AMMONIA OXIDIZING BACTERIA

By

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MOLECULAR METHODS TO DETECT REGULATORY GENES IN ANAEROBIC AMMONIA OXIDIZING BACTERIA

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This project is a collaborative effort between the University of Arizona, Oklahoma State University, and Pima County Reginal Wastewater Reclamation Department (PCRWRD).

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Title of Study: MOLECULAR METHODS TO DETECT REGULATORY GENES IN ANAEROBIC AMMONIA OXIDIZING BACTERIA

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Abstract:

Anaerobic ammonia oxidizing bacteria (anammox) are a group of bacteria capable of using nitrite (NO_2^-) as their electron acceptor and ammonium (NH_4^+) as their electron donor to produce Nitrogen gas (N_2) in anaerobic environments. This is the ANAMMOX reaction. In this process, there is a 50-60% reduction in energy requirements, less sludge production, and does not require BOD compared to traditional nitrogen removal processes. The ANAMMOX discovery is credited to the work of Dr. Arnold Mulder who found that decreasing ammonium and nitrite concentrations were coupled with the release of N_2.

In this study, enriched anammox granules were bioaugmented into single stage partial nitration/ANAMMOX (PN/A) reactors. Acetate was added to the influent of one reactor to simulate the stress of mainstream application of ANAMMOX and to use metagenomic and 16S rRNA analysis to investigate the microbiol community of: 1. Enriched anammox granules, and 2. Anammox bacteria in PN/A reactors subjected to acetate and non-acetate stress. In both cases, the metagenome was mapped, phylogenic trees were developed, and 16S rRNA genes were analyzed using qPCR methods.

Three partial sequences recovered from the metagenome needed to be aligned to form a full-length consensus hydrazine oxidoreductase sequence. The consensus sequence has a high potential to serve as a template for new reverse transcription quantitative PCR (RT-qPCR) based biomarker for anammox activity in future studies.

A qPCR analysis shows that the abundance of *C. Brocadia* and *C. Kuenenia* as well as all other anammox bacteria and all members of *Planctomycetes* were lower in acetate stressed reactors (reactor 2) than in reactor 1. This is also true for the Hzo gene. This indicates that acetate stress will reduce the numbers of anammox bacteria within the time frames of this study.

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CHAPTER I

Introduction

1.1 Problem Statement

Excess Nitrogen (N) in wastewater is a concern because ammonia (NH_3) is toxic to aquatic life. The concentration of NH_3 is dependent on the total concentration of ammonium (NH_4^+), pH, and temperature (Brezonik, et al., 2011). For this reason, discharge permits for wastewater treatment plants (WWTPs) have become stricter on the amount of nutrient nitrogen in the waste stream. Nitrogen loading from watersheds and atmospheric deposition have doubled the rate of N input into estuaries and coastal oceans since the industrial and agricultural revolutions. Modeling by the United States Geological Survey estimates that 4.8 Terra grams (Tg) of N per year was introduced into river systems and that coastal zones, inlands, and drylands had a flux of 7.0 Tg of N per year from river runoff (Suddick et. al. 2013). Adverse effects of increased N loading are eutrophication, acidification, biodiversity loss, and economic costs to aquatic industries that rely on clean water like fisheries and tourism. Almost all freshwater and coastal zones are negatively affected by N loading (Suddick et al. 2013; Howarth et. al. 2011). Human activities have increased the availability of reactive nitrogen in many ecosystems.

The trends of increased available nitrogen in ecosystems have led to negative impacts on human health, biodiversity, and water quality (Finlay, et al., 2013; Kartal et al, 2010).

1.2 Background

This project is a collaborative effort between the University of Arizona, Oklahoma State University, and Pima County Reginal Wastewater Reclamation Department (PCRWRD). The overall objective is to determine if highly enriched anammox granules from the side-stream wastewater flow (i.e. digester supernatant) can be implemented into the mainstream wastewater flow so that simultaneous nitration/ANAMMOX can remove more substantial amounts of N. The project revolves around the cultivation of enriched anammox granules, bioaugmentation of the mainstream waste flow with the granules, metabolic and kinetic characterization, and the metagenomic, metatranscriptomic, and 16S rRNA gene evaluation of anammox performance. This study focuses on metagenomic, metatranscriptomic, and 16S rRNA gene evaluation of anammox performance.

The PCRWRD oversees the wastewater treatment of Pima County and treats 63 MGD of wastewater. The average ammonium concentration of influent wastewater is 34 mg/L as ammonium. The largest facility, Tres Rios, treats all regional biosolids and generates the side stream waste flow of 0.5 MGD with 1000 mg/L of N as ammonium. This side stream accounts for 19 % of nutrient N load while accounting for just 0.8 % of the total volume. These values are similar to other WWTPs.

Current processes of N removal has high-energy requirements and utilizes the biological treatment process of nitrification-denitrification. In this current two-step

process, ammonia is first fixed to nitrite and nitrate using nitrifying bacteria and aeration systems to oxidize influent ammonia. Then, nitrate is used by denitrifying bacteria in anoxic conditions to produce molecular N (N_2). This process requires large amounts of energy and oxygen since all N must be oxidized completely to be removed, produces large amounts of sludge and carbon dioxide, requires separate aerobic and anoxic reactors, and requires biological oxygen demand. However, an emerging technique to better deal with nutrient N in wastewater effluent is the use of ANaerobic AMMonia OXidazion (ANAMMOX). The discovery of the ANAMMOX reaction is credited to the work of Dr. Arnold Mulder who found that decreasing ammonium and nitrite concentrations were coupled with the release of N_2 (Kallistove et. al. 2016; Kartal et. al. 2013). ANAMMOX reactions represent one of the most energy efficient process in wastewater treatment (Christopher et. al. 2017). The ANAMMOX reaction is carried out by a specific subset of anaerobic ammonia oxidizing bacteria (anammox). Anammox bacteria use nitrite as their electron acceptor, and ammonia as their electron donor to produce N_2. In this process, there is a 50-60% reduction in energy requirements, less sludge production, and does not require BOD.

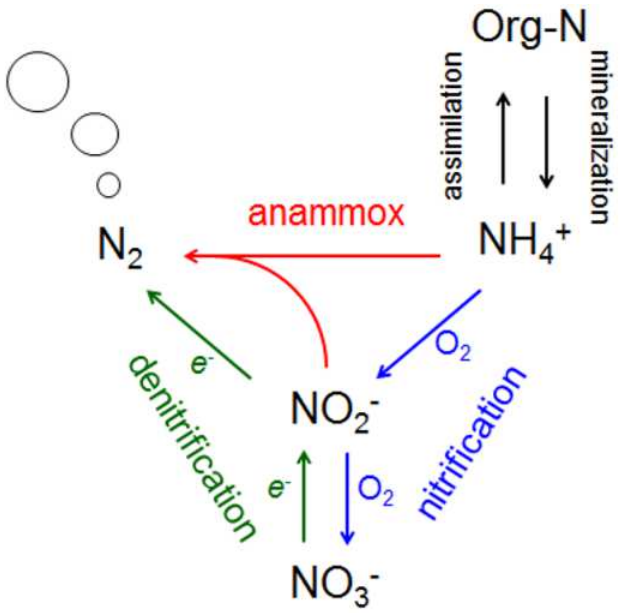

Figure 1.2.1 Schematic of traditional ammonia removal contrasted with ANAMMOX ammonia removal.

1.3 ANAMMOX Process, Metabolism, and Literature Review

ANAMMOX is responsible for an estimated 50% of nitrogen turnover in aquatic environments. The ANAMMOX process has been documented in many diverse marine environments ranging from low to high concentration of salinity and temperatures (Kartel et al. 2010; Grismer, Collison. 2017). The chemical conversion of ammonia and nitrite to molecular nitrogen takes place in the anammoxosome, a membrane bound organelle within the cell. The first step in anammox metabolism of ammonium to molecular nitrogen is the formation of nitrogen monoxide (NO) by nitrite reductase (NirS). Second, NO is condensed with ammonium to form hydrazine (N_2H_4), a mutagenic intermediate. The formation of hydrazine is a unique feature of anammox bacteria with no other known homologs and is catalyzed by hydrazine synthase (HZS). The final step is the oxidation of hydrazine to molecular nitrogen. This process is done by the hydroxlamine

oxidoreductase (HAO)-like enzyme termed hydrazine dehydrogenase (HDH) (also called hydrazine oxidoreductase (Hzo)) (Kallistova et al., 2016; Kartal et al., 2013; Park et al. 2010).

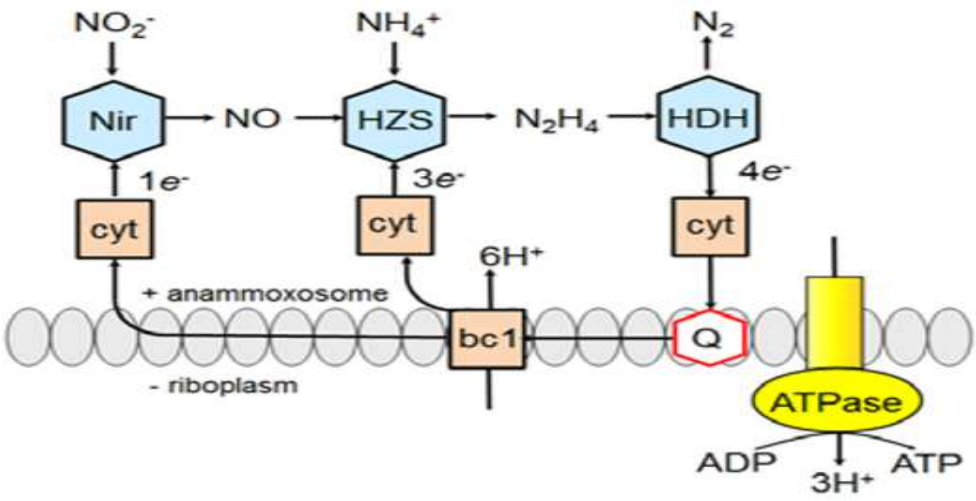

Figure 1.3.1 Hypothesized Anammox Metabolism

These key, enzyme linked, metabolic mediators make suitable targets to study anammox activity and the up/down regulation of anammox genes.

However, ANAMMOX use has its limitations. Anammox bacteria have slower growth rates compared to their denitrifiers-nitrifiers counterparts, having a doubling time of 1-2 weeks (Wang et al 2016, Kallistove et al. 2016; Kartal et al. 2013). Anammox slow growth rates limit their use in carbon rich waste flows because other heterotrophic bacteria quickly outcompete them for required nutrients. Furthermore, it has been shown in a substrate toxicity test that ANAMMOX reactors are negatively affected by high nitrite toxicity (Strous et al. 1999, Fernández et al. 2012). Other limiting factors include increased salinity, carbonaceous oxygen demand, heavy metals, and high organic matter concentrations (Kartal et al. 2006, Chamchoi et al. 2008, Molinuevo et al. 2008, Ni et al.

2012, Li, et al., 2014). These limitations must be considered when designing N removal by anammox bacteria.

Regardless of anammox's inherently finicky metabolism, over 110 full-scale ANAMMOX reactors are being used world-wide to treat NH_4^+ rich waste streams (Mao et al. 2017). There are two popular reactor methods to achieve the proper ratio of NO_2^- and NH_3 loading. One is single stage partial nitration/ANAMMOX (PN/A) and the second is two stage PN/A reactors. Figure 1.3.2 shows a proposed design in using anammox granules to be bioaugmented into the mainstream waste flow (courtesy of James Field, University of Arizona).

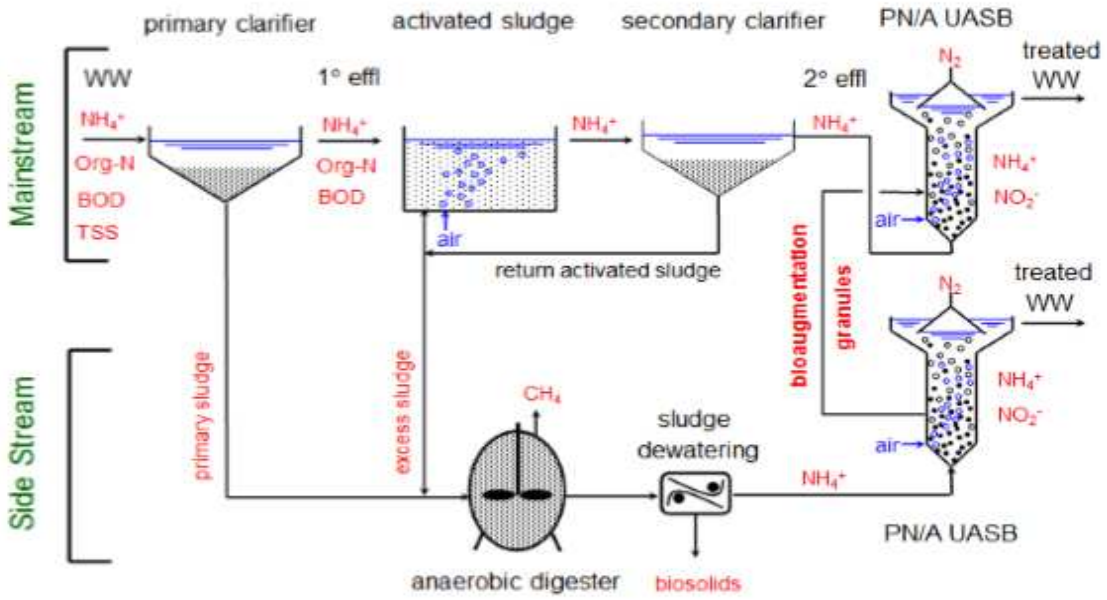

Figure 1.3.2 Proposed ANAMMOX Treatment Train

In single stage PN/A reactors, nitration is used simultaneously with ANAMMOX to produce nitrite for ANAMMOX reactions in the same reactor. In the latter, a second

reactor is dedicated to the nitration process to produce NO_2^-, which is then the influent to the ANAMMOX reactor. The use of two stage PN/A reactors yields greater control of the influent nitrite and ammonia concentrations. An application survey found that 50% of all PN/A reactors were sequence batch reactors, and that 88% of all plants were being operated as a single stage PN/A reactor, 75% of which treat the side stream waste flow (Lackner et al. 2014). Furthermore, in a lab scale comparison, full nitration-denitration was compared to PN/A reactors. It was found that the PN/A reactors had a 40% increase in total nitrogen removal (Zhou et al. 2018).

1.4 ANAMMOX Genomes and Microbial Community Characteristics

Five genera of anammox bacteria have been discovered to date. These are: *Kuenenia*, *Brocadia*, *Anammoxoglobus* and *Jettenia* commonly found in activated sludge, and *Scalindua* commonly found in marine environments. These lineages all have *'Candidatus'* status as they do not exist in pure culture and must be grown and studied as enrichments (Christopher et al. 2017). Furthermore, numerous studies have confirmed that the phyla *Chlorobi, Bacteroidetes, Chloroflexi*, and *Proteobacteria* make up a large portion of microbial cultures across a wide range of ANAMMOX reactors (Christopher et. al. 2017). Genomes affiliated with *Brocadia* (AMX1) and *Chlorobi* (CHB1) dominated the abundance and gene expression of the microbial community in anammox granules. Other organisms that displayed moderate abundance and gene expression in the anammox ecosystem were affiliated with the phyla *Chlorobi, Bacteroidetes, Chloroflexi* and *Proteobacteria* (Christopher et. al. 2017).

Anammox seed granules harvested from a laboratory-scale up-flow anaerobic sludge blanket (UASB) reactor were used for batch and continuous-flow experiments using synthetic wastewater in phosphate toxicity test (Zehand et. al. 2017). The most abundant bacterial phyla were *Planctomycetes. Planctomycetes* accounted for 47.6% (control) and 40.4% (phosphate) of the biomass, with mainly classes of *Brocadiae* and *Phycisphaerae*. Although the class *Phycisphaerae* does not belong to anammox bacteria, it could be contributing to the formation of anammox granules. The family *Brocadiaceae* was found to have an abundance of 40.3% and 13.4% of all bacteria in control and phosphate samples respectively. Specifically, the genus *Candidatus Kuenenia (C. Kuenenia)* were at a concentration of 99.9% and 99.8% of all anammox bacteria in control and phosphate samples respectively. Other anammox bacteria consisted of species of *Candidatus Jettenia* (*C. Jettenia*) and *Candidatus Brocadia* (*C. Brocadia*). The Phyla of *Proteobacteria* was the second most abundant and *Chloroflexi* were third most dominant with an increase of 10.3% to 15.6% after phosphate addition. *Chloroflexi* play an important role in sludge granulation process by forming the initial framework for small sludge particles, and therefor likely play an important role in anammox granules. *Bacteroidetes* may also contribute to enhance granules by forming the construction of web-like structures in the outer layer of sludge granules.

In a nitrite study using an enrichment culture taken from a lab scale anammox/DAMO reactor, which was fed with ammonium, nitrite and methane, it was found that *Kueneniaceae* comprised only 0.6% of the community while *M. nitroreducens* comprised 70%. In nitrite fed reactors, *M. nitroreducens (*26%) increased in cluster size and was surrounded by anammox bacteria suggesting synergistic interactions between the

two populations. 2.6% of the total community was comprised of *Kueneniaceae* (Hu. Et. al. 2015).

In two stage PN/A reactor lab scale test, fed anaerobic digester filtrate from the belt press of a local WWTP, it was shown that the ANAMMOX reactors could sustain 82% total N removal in both phases (start-up and two stage PN/A). Using FISH, the microbial community was shown to consist mainly of *Kuenenia stuttgartiensis* (65%) along with several unidentified potential anammox strains in the ANAMMOX reactor. The nitration reactor was shown to have a dominating species of *Nitrosomonas europaea* (Kotay et al. 2012).

In anammox granulation studies using reactors that were fed partially nitrified anaerobic digestion, it was found that anammox concentrations were variable. Showing a range from $8x10^6$ to $5.5x10^8$ copies/mL before granulation. After anammox granules were established, anammox concentrations increased and stabilized to a range of 0.4 to $7.8x10^8$ copies/mL (Park et al. 2010).

CHAPTER II

Methods and Materials

2.1 Sampling and Reactor Set-up

Enriched anammox granules were used as the seed sludge to bioaugment two, single stage, partial nitration/ANAMMOX (PN/A) reactors. The PN/A reactors in this experiment were kept under prime conditions to promote anammox growth at the University of Arizona. Both were fed municipal wastewater from PCRWRD with the exception that reactor 1 was fed the optimal nutrient loading to satisfy anammox bacteria; and reactor 2 was fed increased amounts of acetate to mimic the main waste stream application of ANAMMOX. Fifteen 1.5 mL sample tubes from reactors 1 and 2, and the seed sludge, dated December 2018 and January 2019, were received from the University of Arizona in the spring of 2019, well frozen, and were stored at -80 °C.

2.2 Nucleic Acid Extraction

RNA was extracted from five samples from each of the reactors. A dedicated biological safety hood for RNA work was used to limit contamination of samples. The work area, including tips, pipettes, and reagents were exposed to UV light for a period of

30 minutes or longer. RNase-free DNase and ethanol were then used to wipe down the work area and gloves when coming back into contact with the work area. All movement out/in the work area was done so to minimize disturbance of the air blanket of the biological safety hood. RNA PowerSoil® Total RNA Isolation Kit (Qiagen, Hilden, Germany) was used to isolate RNA in accordance with manufacture protocol.

DNA was extracted from one sample from each reactor, plus the seed sample. DNA extraction work was carried out on an open lab bench, away from drafts, using aseptic techniques. Dneasy PowerSoil® Kit (Qiagen, Hilden, Germany) was used to extract DNA in accordance with manufacture protocol. All nucleic acid extracts were stored at -80 °C until downstream applications; during which all samples were kept in an ice bath.

2.3 Nucleic Acid Quantification and Purity

RNA and DNA samples were quantified for total RNA and DNA presence using a Quantifluor RNA and Quantifluor dsDNA Systems (Promega, Madison, Wisconsin), respectively. These systems were used in accordance with manufacturer protocols. RNA work was done in the dedicated RNA work area described previously; and the low quantity RNA standards were prepared and used in RNA quantifications. All samples were then incubated at room temperature for 5 minutes and florescence was measured using the Quantus Fluorometer (Promega, Madison, Wisconsin) with a 2 µL sample.

Using a NanoDrop OneC (Thermo Fisher, Waltham, Massachusetts), the samples were tested for protein and organic compound contamination. Elution buffers from each nucleic acid extraction kit was used as the blank. Nucleic Acids, both DNA and RNA,

absorb UV light at 260 nm. In contrast, proteins and organic compounds absorb light at 280 nm and 230 nm respectively. Therefore, the ratio of absorption at 260/280 and 260/230 gives the relative purity for protein and organic compound contamination. A highly pure DNA or RNA sample is expected to produce a 260/280 ratio above 1.8 and 2.1 respectively. A low ratio of 260/280 means there was a high percentage of absorption in the UV range of proteins and indicates protein contamination. The same is true for the 260/230 ratio. Pure samples are expected to have a ratio near 2. Any ratio lower than 1.8 is considered highly contaminated with organic compounds.

2.4 Primer Selection

Primer pairs were selected to target all anammox bacteria, the phylum *Planctomycetes,* anammox species of *Ca. Kuenenia and Brocadia* (16S rRNA) and the anammox gene Hydrazine Dehydrogenase (functional gene). Primers were manufactured by Thermo Fisher Scientific (Waltham, Massachusetts) and were diluted to 20 µM for a working stock. Table 2.4.1 details the primer target, *E. coli* position, annealing temperature, and reference to cited literature.

Table 2.4.1 Primer Pairs, Targets, and Annealing Temperatures

Primer Pair	Target	BP Length	Annealing Temperature	Reference
A438F/A684R	All anammox bacteria	248	55 °C	Sonthiphand et al. 2013, Humbert 2012
Amx368F/820R	*Ca. Kuenenia, Ca. Brocadia*	830	59 °C	Sonthiphand et. al. 2013
HzoCl1F1/HzoCl1r2	Hydrazine dehydrogenase	470	47 °C	Wang et. al. 2016, Schmid 2008
Pla46F/1392R	*Planctomycetes*	1400	55°C	Sonthiphand et. al. 2013, Schmid 2000

2.5 RNA and DNA Metagenomic, Metatranscriptomic, and 16S rRNA Gene Evaluation

Metagenome sequence from anammox biofilms in P/NA reactors. Aliquots of the DNA extractions from Reactors 1 and 2 from both December and January, and the seed

sludge were all pooled together at a concentration of 1000 ng/µL and a total sample volume of 80.9 µL ensuring that equal mass amounts of DNA were added. Pooled DNA extracts, and representative RNA extracts were transferred to an autoclaved 1.5mL microcentrifuge tube, wrapped in parafilm, placed in a paper towel, sealed in a zip-lock bag, and placed beneath dry ice in an approved shipping container prior to overnight shipment to Novogene Pharma, Sacramento California for genomic sequencing analysis. Results were analyzed with help from Drs. Couger and Krzmarzick (Oklahoma State University).

Metagenome sequencing from Anammox Granules. DNA extract (37 ng/µL) was sent to Molecular Research (MRDNA) (Shallowater, TX) for metagenomic sequencing using 2x150bp paired sequences (20 million reads scale) on an Illumina HiSeq system (Illumina, San Diego, California). At MRDNA, the library was prepared using Nextera DNA sample preparation kit (Illumina, Sad Diego, California) with 50 ng of DNA. The sample underwent simultaneous fragmentation with the addition of adapter sequences. These adapters were utilized during a 5-cyle PCR in which unique indices were added to the sample. The final concentration (7.54 ng/µL) was then determined with Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, California) and the average library size (1047 bp) was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California). The library was diluted to 10 pM and sequenced paired ends for 300 cycles using a HiSeq system (Illumina, San Diego, California).

Metagenomic assembly. Metagenome assembly was performed by Dr. Brian Couger. 16S rRNA sequences and selected functional genes from the genomes were recovered and Phylogenetical analysis of the genomes and a classification table was produced.

Phylogenetic Analysis. Phylogenetic analysis was performed in MEGA. The 16S rRNA genes were analyzed using NCBI nBLAST, with all non-cultured sequences taken out of the analysis. The most identical match was recovered and the 16S rRNA sequences were aligned using MUSCLE. For functional genes, the amino acid sequences were used to recover most similar homologous sequences from BLAST, and alignment was performed on the amino acid level with MUSCLE. For Phylogenetic analysis in all cases, evolutionary histories were inferred using the Neighbor-Joining method (Saitou and Nei. 1987) with a bootstrap analysis (Felsenstein 1985) with 1000 bootstraps. The phylogenic trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, Kumar 2004) and are in the units of the number of base substitutions per site. Analyses were conducted in MEGA6 (Tamura et. al. 2013).

2.6 PCR Protocol, Gel Electrophoresis, and Gel Imaging

Polymerase Chain Reaction (PCR) master mix consisted of 31.35 µL PCR water, 0.2 mM 5x Colorless GoTaq Flexi Buffer, 0.05 mM BSA, 1 mM $MgCl_2$, 0.08 mM dNTPs, 0.025 mM Gold Taq Polymerase and 0.025 mM of each primer appropriate for the assay. Table 2.6.1 details the PCR master mix. In triplicate, 1 µL of each sample was

added to a PCR tube and washed down with 49 µL of PCR master mix. DNA concentrations ranged from 39 to 82 ng/µL and are detailed in section 4 of the results chapter. A BioRadT100 Thermo Cycler (Bio-Rad, Hercules, California) was used with the following thermo profile: initiation with 95 °C for 3 minute, followed by 35 cycles of: 95 °C for 30 seconds, annealing temperature for 1 minute, and extension at 72 °C for 1 minute, and a final extension step at 72 °C for 7 minutes. PCR products were then loaded into 1.75% high resolution agarose gel (Sigma Aldrich, Darmstadt, Germany) that had been pre-casted with GelRed Nucleic Acid Stain (Biotium, Fremont, California). Using a PuroGEL Electrophoresis System (Luna Nanotech, Toronto, Ontario), the gel was subject to 50 volts for 50 minutes and then chilled (4 °C) before gel imaging was conducted on a Gel Doc™ XR+ with Image Lab™ Software (Bio-Rad, Hercules, California).

Table 2.6.1 PCR Master Mix

Reagent	Concentration in Master Mix	Manufacturer
5x Colorless GoTaq Flexi Buffer	0.2 µM	Promega
BSA	0.05 µM	Made in Lab
$MgCl_2$, 25mM	1 mM	Promega
d NTP Mix, 10mM	.08 mM	Thermo Fisher Scientific
GoTaq G2 Flexi DNA Polymerase, 5 u/µL	.025 units/µL	Promega
Forward Primer	0.25 mM	Thermo Fisher Scientific
Reverse Primer	0.25 mM	Thermo Fisher Scientific
Molecular Grade Water		MoBio

- Reagents, mM concentrations, and product manufactures are listed.

2.7 qPCR Thermo Profiles

Qualitative Polymerase Chain Reaction (qPCR) master mix for qPCR consisted of 1× Syber Green Mix, 5 µg/µL BSA, 300 nM of each primer in 10 µL total volume. The thermo cycle was initiated with 5 min denaturing at 95°C, followed by 45 cycles of: denaturing at 95 °C for 15 seconds, annealing at assigned temperature for 60 seconds,

and extension at 75 °C for 30 seconds, with a final extension step of 75 °C for 7 minutes. Fluorescent signal was measured after each completed cycle and a melting curve analysis was performed to ensure primer specificity (Wang et. al. 2016; Harhangi et al. 2012).

2.8 qPCR Standard Protocol

Standards for anammox 16S rRNA and functional genes were developed using the pGEM-T Easy Vector System II (Promega, Madison, Wisconsin). First, DNA extracts were subject to qPCR. Samples with the highest fluorescence were selected for a follow-up PCR to ensure sticky ends were added to the gene segment. Then, PCR products were washed using an Ultraclean PCR Clean-Up Kit (Qiagen, Hilden, Germany) following manufacture protocol. Lyses blue was added to the reagents as instructed by the manufacture.

Immediately, PCR products were ligated at 4°C for 18 hours (Promega, Madison, Wisconsin). 3 µL of cleaned PCR product was washed down a PCR tube with 7 µL of ligation mix. Table 2.8.1 details the ligation mix.

Table 2.8.1 Ligation Mixture

Reagent	mM Concentration	Manufacture
2x Rapid Ligation Buffer	1x	Promega
pGEM-T Easy Vector, 50 ng/μL	0.005 mM	Promega
T4 DNA Ligase, 3u/μL	0.3 Weiss units mM	Promega

The ligation products were then used in the cloning and transformation process. JM109 competent cells were thawed on an ice bath, tips and tubes were frozen at -20 °C, and the pipettes and work surface were sanitized with 70% ethanol before work began. 7 μL of ligation product was added directly to 50 μL of JM109 competent cells and allowed to incubate in the ice bath for 30 minutes. Next, the cells were incubated in a warm water bath set to 42 °C for 90 seconds. Immediately following the warm water bath, cells were placed back into the ice bath for 2 minutes. 800 μL of lab made SOC media (table 2.8.2) was then added to the cell cultures and allowed to incubate at 37 °C for 30 minutes.

Table 2.8.2 SOC Media

Reagent	Quantity (g)	Manufacture
Tryptone	20	Sigma-Aldrich
Yeast Extract	5	Thermo Fisher Scientific
NaCl	0.58	Thermo Fisher Scientific
KCl	0.19	Thermo Fisher Scientific
$MgSO_4$	1.21	Thermo Fisher Scientific
$MgCl_2*6H_2O$	2.03	Thermo Fisher Scientific
D-glucose	3.60	Thermo Fisher Scientific
RO Water	1 L	

- Solution was autoclaved for a period of 3 hours.

200 µL of transformed cells were then plated on pre-made LB/Ampicillin/IPTG/XGAL plates (see section 2.9 for selective growth media preparation). Plated cells were incubated over night at 37 °C for 18 hours. A single white colony was selected to be inoculated into 100 mL of LB broth and incubated for 18 hours at 37°C. Incubated cells were then quadrant streaked onto fresh LB/Ampicillin/IPTG/XGAL plats to ensure plasmid purity and to increase plasmid concentration. Again, a single white colony was selected to be inoculated into 100 mL of fresh LB broth and incubated as previously described.

In triplicate, using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), 5 mL of incubated cell culture was centrifuged at 4,000 rpm (4x Gravity) for 1 minute and

subjected to manufacture protocols to extract the plasmid. Triplicate plasmid extracts were pooled together once completed. QuantiFluor dsDNA System (Promega, Madison, Wisconsin) was then used to quantify the plasmid concentrations. A working stock of each plasmid was created by serial dilution from 10^{-1} to 10^{-8} and stored at -20 °C. Undiluted plasmid stock was stored at -80 °C. In order to keep a stock of cell culture, 10 mL of cultured transformed cells were placed into 10 mL of glycerol, mixed well, and stored at -80 °C.

2.9 Selective Growth Media

In order to selectively grow JM109 cells that had successfully up took the desired plasmid, LB/Ampicillin/IPTG/XGAL plates were prepared fresh before each transformation. LB broth consisted of 15 g Tryptone (Sigma Aldrich, Darmstadt, Germany), 10 g yeast extract (Thermo Fisher Scientific, Waltham, Massachusett), and 10 g NaCl. A portion of this broth was saved for the incubation step previously mentioned from section 2.8. To make the agar plates, 15 g of agar (Fisher Scientific, Waltham, Massachusetts) was added per 1 L of LB broth and allowed to dissolve. The solutions were then autoclaved for a period of 3 hours and allowed to cool. Once warm to the touch, 200 mg/L of Ampicillin Sodium Salt (Fisher Scientific, Waltham, Massachusetts) was added to the LB agar solution and allowed to mix. The solution was then poured into petri dishes and allowed to solidify.

100 mL of ChromoMax IPTG/X-GAL Solution (Fisher Scientific, Waltham, Massachusetts) were added to solidified LB/Ampicillin plates. The IPTG/XGAL solution

was evaporated from the plate surface for no less than 1 hour and stored at 4 °C for no longer than 24 hours before use. Stored LB/Ampicillin/IPTG/XGAL plates were then allowed to warm to 37 °C before transformed cell cultures were added.

CHAPTER III

Results

3.1 Nucleic Acid Extract Concentration and Purity

Results from RNA and DNA extract quantifications are shown in tables 3.1.1 and 3.1.2 respectively. All samples (5 from each reactor for RNA extracts and 3 from each reactor for DNA extracts) have relative high concentrations and are shown in ng/µL of extract.

Table 3.1.1 RNA Quantification Results

Sample	Conc. ng/uL
Standard	5
Reactor 1 (RNA)	28
Reactor 1 (RNA)	28
Reactor 1 (RNA)	27
Reactor 1 (RNA)	21
Reactor 1 (RNA)	26
Reactor 2 (RNA)	18
Reactor 2 (RNA)	15
Reactor 2 (RNA)	19
Reactor 2 (RNA)	20
Reactor 2 (RNA)	18

Table 3.1.2 DNA Quantification Results

Sample	Conc. ng/µL
Standard	100
Reactor 1 December (DNA)	54
Reactor 2 December (DNA)	75
Reactor 1 January (DNA)	47
Reactor 2 January (DNA)	56
Seed (DNA)	101

Purity of RNA and DNA extracts are shown below in table 3.1.3. A measurement greater than 2 is considered a highly pure sample. See section 2.3 for nanodrop details.

Table 3.1.3 Nano Drop Results

Sample	A 260/280	A 260/230	ng/uL
Reactor 1 RNA	2.19	2.39	122
Reactor 2 RNA	2.13	2.60	80.5
Combined DNA	1.94	3.19	47

- Ratios and concentrations show good purity in all samples.

3.2 Genomic Results from Anammox Granules

From the anammox granules, a total of 13 nearly complete genomes were recovered. The 16S rRNA genes were recovered and analyzed via a classification software and a phylogenetic tree of the metagenomes 16S rRNA sequences, and the most

identical 16S rRNA genes from NCBI's Blast searches are shown if figure 3.2.1. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site.

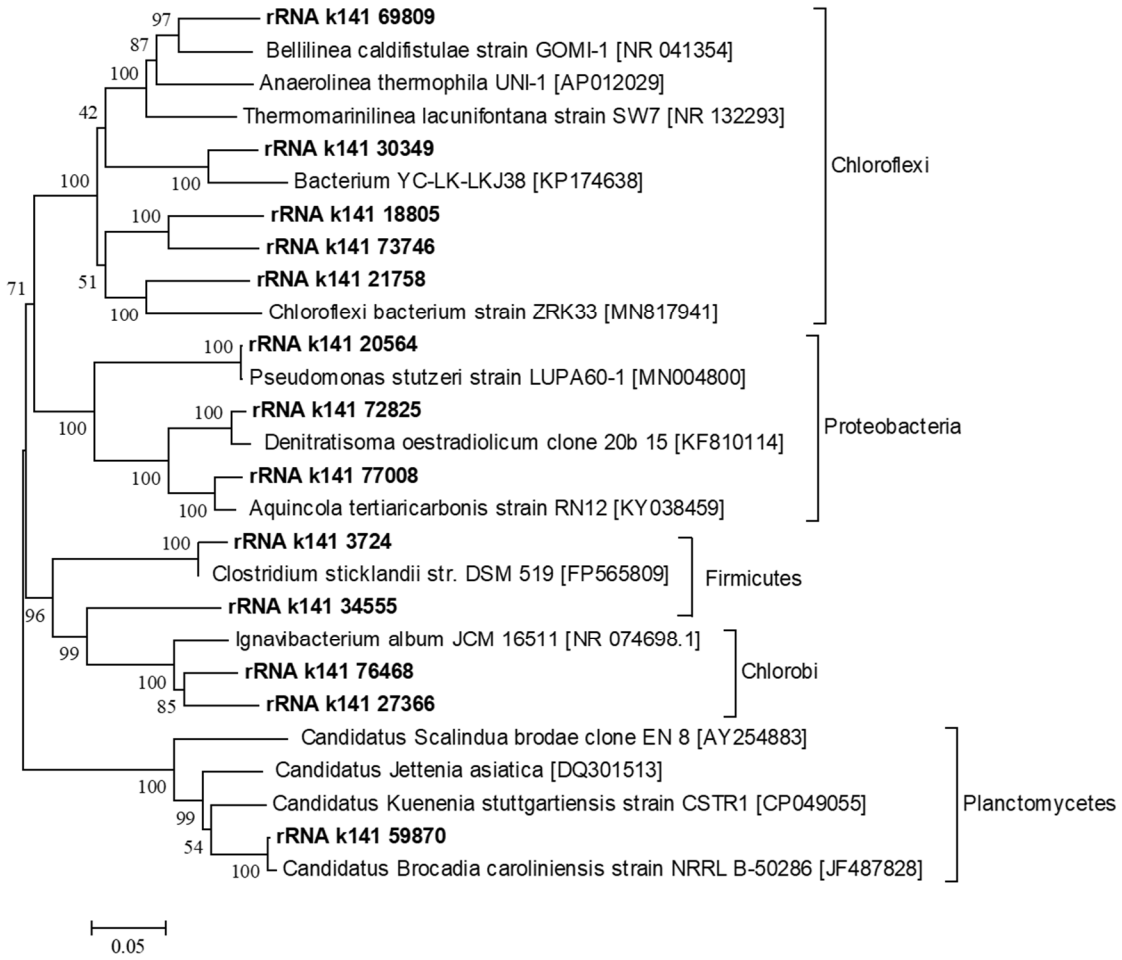

Figure 3.2.1. 16S rRNA gene base phylogenetic analysis of the genomes recovered from the anammox granules.

From the anammox granule metagenome analysis, the phylogenic classifications of the 13 most complete genomes are shown.

Table 3.2.1. Phylogenetic Classification of Recovered Genomes from Anammox Granules.

Genome designation	*Phylum*	*Class*	*Order*	*Family*	*Genus*
k141_76468	*Chlorobi*	*Ignavibacteria*	*Ignavibacteriales*	*Ignavibacteriaceae*	*
k141_69809	*Chloroflexi*	*Anaerolineae*	*envOPS12*	*	*
k141_20564	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
k141_18805	*Chloroflexi*	*Anaerolineae*	*SBR1031*	*SHA-31*	*
k141_73746	*Chloroflexi*	*Anaerolineae*	*SBR1031*	*A4b*	*
k141_3724	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptostrepto-coccaceae*	*
k141_27366	*Chlorobi*	*Ignavibacteria*	*Ignavibacteriales*	*Ignavibacteriaceae*	*
k141_21758	*Chloroflexi*	*Anaerolineae*	*SBR1031*	*A4b*	*
k141_34555	*Firmicutes*	*Clostridia*	*Clostridiales*	*	*
k141_59870	*Planctomycetes*	*[Brocadiae]*	*Brocadiales*	*Brocadiaceae*	*Candidatus Brocadia*
k141_72825	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Dok59*
k141_30349	*Chloroflexi*	*Anaerolineae*	*S0208*	*	*
k141_77008	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*

3.3 Metagenomic Results from PN/A Reactors 1 and 2 and the Seed Sludge

From the PN/A biofilm metagenomic sequencing, 77 total nearly complete genomes have been identified (i.e. over 80% estimated genome coverage). Table 3.3.1 shows the genomic classification of all genomes with limits of 80% completeness and 10% contamination. One complete genome has no known genomes within its phylum in the NCBI database, and thus represents potentially a novel bacterial phylum. 17 phyla were mapped from the DNA samples. The phylum of *Planctomycetota* houses the bacteria known to be capable of the ANAMMOX reaction. Specifically, *Brocadia caroliniensis,* which was found in bin 111 with 94.51% completeness and 2.75% contamination.

Table 3.3.1 Phylogenic Classification of Recovered Genomes from Reactors 1, 2, and the Seed Sludge.

Bin #	Phylum	Class	Order	Family	Genus	Species	% Completeness	% Contamination
Bin 176	*Bacteroidota*	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*Ferruginibacter*	*Ferruginibacter sp001898465*	99.51	0
Bin 206	*Actinobacteriota*	*Acidimicrobiia*	*IMCC26256*	*	*	*	99.15	1.28
Bin 40	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*UBA2796*	*UBA2796*	*UBA2796 sp002352035*	99.09	3.64
Bin 123	*Hydrogenedentota*	*Hydrogenedentia*	*Hydrogenedentiales*	*	*	*	98.9	1.1
Bin 168	*OLB16*	*OLB16*	*OLB16*	*OLB16*	*	*	98.88	3.37
Bin 129	*Planctomycetota*	*Phycisphaerae*	*Phycisphaerales*	*SM1A02*	*UBA2396*	*	98.86	1.14
Bin 118	*Actinobacteriota*	*Acidimicrobiia*	*UBA2766*	*	*	*	98.29	2.14
Bin 94	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*UBA2029*	*	*	97.98	0.91
Bin 112	*Planctomycetota*	*Phycisphaerae*	*UBA1845*	*UBA1845*	*	*	97.73	2.27
Bin 72	*Planctomycetota*	*Phycisphaerae*	*UBA1845*	*Fen-1342*	*	*	96.59	1.14
Bin 83	*Proteobacteria*	*Alphaproteobacteria*	*Dongiales*	*Dongiaceae*	*	*	96.51	2.74
Bin 70	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*	*	96.37	1.8
Bin 85	*Bacteroidota*	*Bacteroidia*	*Flavobacteriales*	*PHOS-HE28*	*PHOS-HE28*	*	96.34	6.99
Bin 19	*Acidobacteriota*	*Acidobacteriae*	*Bryobacterales*	*Bryobacteraceae*	*	*	95.89	1.32
Bin 162	*Planctomycetota*	*Phycisphaerae*	*Phycisphaerales*	*SM1A02*	*	*	95.45	0
Bin 5	*Planctomycetota*	*Phycisphaerae*	*UBA1845*	*Fen-1342*	*	*	95.45	1.14
Bin 82	*Planctomycetota*	*UBA8742*	*UBA2392*	*UBA2392*	*UBA2392*	*UBA2392 sp002343805*	95.45	7.95
Bin 51	*Bacteroidota*	*Kapabacteria*	*Kapabacteriales*	*Kapabacteriaceae*	*OLB6*	*OLB6 sp001567175*	95.36	0.55
Bin 127	*Verrucomicrobiota*	*Verrucomicrobiae*	*Pedosphaerales*	*Pedosphaeraceae*	*	*	95.27	0.68
Bin 207	*FEN-1099*	*FEN-1099*	*	*	*	*	95.16	1.94
Bin 92	*Bdellovibrionota*	*Bacteriovoracia*	*	*	*	*	94.64	2.68
Bin 111	*Planctomycetota*	*Brocadiae*	*Brocadiales*	*Brocadiaceae*	*Brocadia*	*Brocadia caroliniensis*	94.51	2.75

Bin 171	*Proteobacteria*	*Alphaproteobacteria*	*Ferrovibrionales*	*Ferrovibrionaceae*	*	*	94.31	8.04
Bin 33	*Bacteroidota*	*Bacteroidia*	*AKYH767-A*	*OLB10*	*OLB10*	*OLB10 sp001567275*	94.29	0.48
Bin 181	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*GA007523*	*	*	94.19	2.37
Bin 57	*Myxococcota*	*Polyangia*	*Polyangiales*	*Polyangiaceae*	*	*	93.98	2.9
Bin 28	*Bdellovibrionota*	*Bacteriovoracia*	*	*	*	*	93.75	0.89
Bin 150	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*A4b*	*OLB15*		93.64	0
Bin 45	*Chloroflexota*	*Anaerolineae*	*4572-78*	*	*	*	93.64	2.38
Bin 21	*Bacteroidota*	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*UBA1930*	*	93.60	0.49
Bin 64	*Bacteroidota*	*Bacteroidia*	*Flavobacteriales*	*Koll-22*	*	*	93.55	0.54
Bin 87	*GWC2-55-46*	*GWC2-55-46*	*GWC2-55-46*	*GWC2-55-46*	*UBA5799*	*	93.55	0.54
Bin 60	*Spirochaetota*	*Leptospirae*	*Leptospirales*	*Leptonemataceae*	*Leptonema*	*Leptonema illini*	95.53	2.53
Bin 113	*Myxococcota*	*Myxococcia*	*Myxococcales*	*Myxococcaceae*	*	*	93.23	0.86
Bin 158	*Proteobacteria*	*Alphaproteobacteria*	*Dongiales*	*Dongiaceae*	*	*	93.01	4.95
Bin 6	*Chloroflexota*	*Anaerolineae*	*Anaerolineales*	*envOPS12*	*UBA12294*	*	92.73	0.91
Bin 73	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*A4b*	*GCA-2702065*	*	92.73	1.82
Bin 179	*Acidobacteriota*	*Acidobacteriae*	*Bryobacterales*	*Bryobacteraceae*	*UBA690*	*UBA690 sp003487005*	92.39	1.74
Bin 166	*Myxococcota*	*UBA9042*	*UBA9042*	*	*	*	92.10	2.9
Bin 68	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*SCN-69-89*	*	91.84	1.83
Bin 147	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*UBA2796*	*	*	91.82	4.7
Bin 157	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*A4b*	*	*	91.82	1.82
Bin 56	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Rhodocyclaceae*	*Azospira*	*Azospira oryzae*	91.57	0.53
Bin 131	*Chloroflexota*	*Anaerolineae*	*UBA4142*	*UBA4142*	*	*	91.52	2.91
Bin 137	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*A4b*	*OLB13*	*OLB13 sp001567485*	91.52	0
Bin 187	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Nitrosomonadaceae*	*Nitrosomonas*	*	91.48	1.97

Bin 193	*Proteobacteria*	*Gammaproteobacteria*	*UBA7966*	*UBA7966*	*	*	91.29	3.57
Bin 37	*Chloroflexota*	*Dehalococcoidia*	*UBA2991*	*UBA2991*	*	*	91.09	0.99
Bin 13	*Proteobacteria*	*Alphaproteobacteria*	*Dongiales*	*Dongiaceae*	*	*	s90.83	0.87
Bin 75	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Pseudorhodoplanes*	*	90.35	1.32
Bin 116	*Desulfobacterota_A*	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio_A*	*	90.34	1.25
Bin 143	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Rhodanobacteraceae*	*Dokdonella*	*	90.17	0.86
Bin 26	*Chloroflexota*	*Anaerolineae*	*Anaerolineales*	*	*	*	90.00	3.64
Bin 148	*Bacteroidota*	*Ignavibacteria*	*SJA-28*	*B-1AR*	*	*	89.87	3.55
Bin 108	*Bacteroidota*	*Bacteroidia*	*Flavobacteriales*	*PHOS-HE28*	*PHOS-HE28*	*	89.78	0
Bin 132	*Planctomycetota*	*Phycisphaerae*	*UBA1845*	*Fen-1342*	*Fen-1342*	*	89.77	0
Bin 91	*Planctomycetota*	*UBA1135*	*UBA2386*	*UBA23 86*	*UBA2386*	*	89.77	1.24
Bin 34	*Patescibacteria*	*Gracilibacteria*	*UBA1369*	*UBA1369*	*PALSA-1335*	*	89.60	0.99
Bin 124	*Chloroflexota*	*Anaerolineae*	*SBR1031*	*A4b*	*	*	89.09	0
Bin 142	*Gemmatimonadota*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*SCN-70-22*	*	89.01	3.3
Bin 125	*Planctomycetota*	*UBA1135*	*UBA2386*	*UBA23 86*	*	*	88.64	1.14
Bin 25	*Planctomycetota*	*Phycisphaerae*	*Phycisphaerales*	*SM1A02*	*	*	88.57	0
Bin 133	*	*	*	*	*	*	87.91	1.1
Bin 90	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Rubrivivax*	*	87.85	1.95
Bin 93	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter_E*	*	86.56	1.36
Bin 177	*Verrucomicrobiota*	*Verrucomicrobiae*	*Opitutales*	*Opituta ceae*	*UBA6669*	*	85.62	0.68
Bin 134	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*JOSHI-001*	*	85.37	2.17
Bin 59	*Chloroflexota*	*Anaerolineae*	*Anaerolineales*	*envOPS12*	*OLB14*	*	85.00	1.82
Bin 130	*Verrucomicrobiota*	*Lentisphaeria*	*	*	*	*	84.78	2.06

Bin 36	*Bdellovibrionota*	*Bdellovibrionia*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Bdellovibibrio*	*	83.68	2.7
Bin 185	*Proteobacteria*	*Gammaproteobacteria*	*GCA-2729495*	*GCA-2729495*	*	*	83.35	2.86
Bin 145	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	*	82.92	6.27
Bin 101	*Bacteroidota*	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*	*	82.02	4.68
Bin 97	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Rhodocyclaceae*	*Thauera*	*	81.89	1.26
Bin 156	*Chloroflexota*	*Anaerolineae*	*Anaerolineales*	*envOPS12*	*OLB14*	*	81.82	1.82
Bin 54	*Chloroflexota*	*Anaerolineae*	*Promineofilales*	*Promineofilaceae*	*Promineofilum*	*	81.07	2.73
Bin 50	*Verrucomicrobiota_A*	*Chlamydiia*	*Parachlamydiales*	*FEN-1388*	*	*	79.9	0

3.4 Metagenomic Data for Hydroxylamine and Hydrazine Oxidoreductases Genes

Figure 3.4.1 shows the phylogenetic tree of the hydroxylamine and hydrazine oxidoreductases genes recovered in the dataset. Thirteen nearly complete amino acid sequences, recovered from the granule metagenome, are shown in the tree in comparison of nearest matches in GenBank from pBlast. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 7.47198601 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 42 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1074 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et. al. 2013).

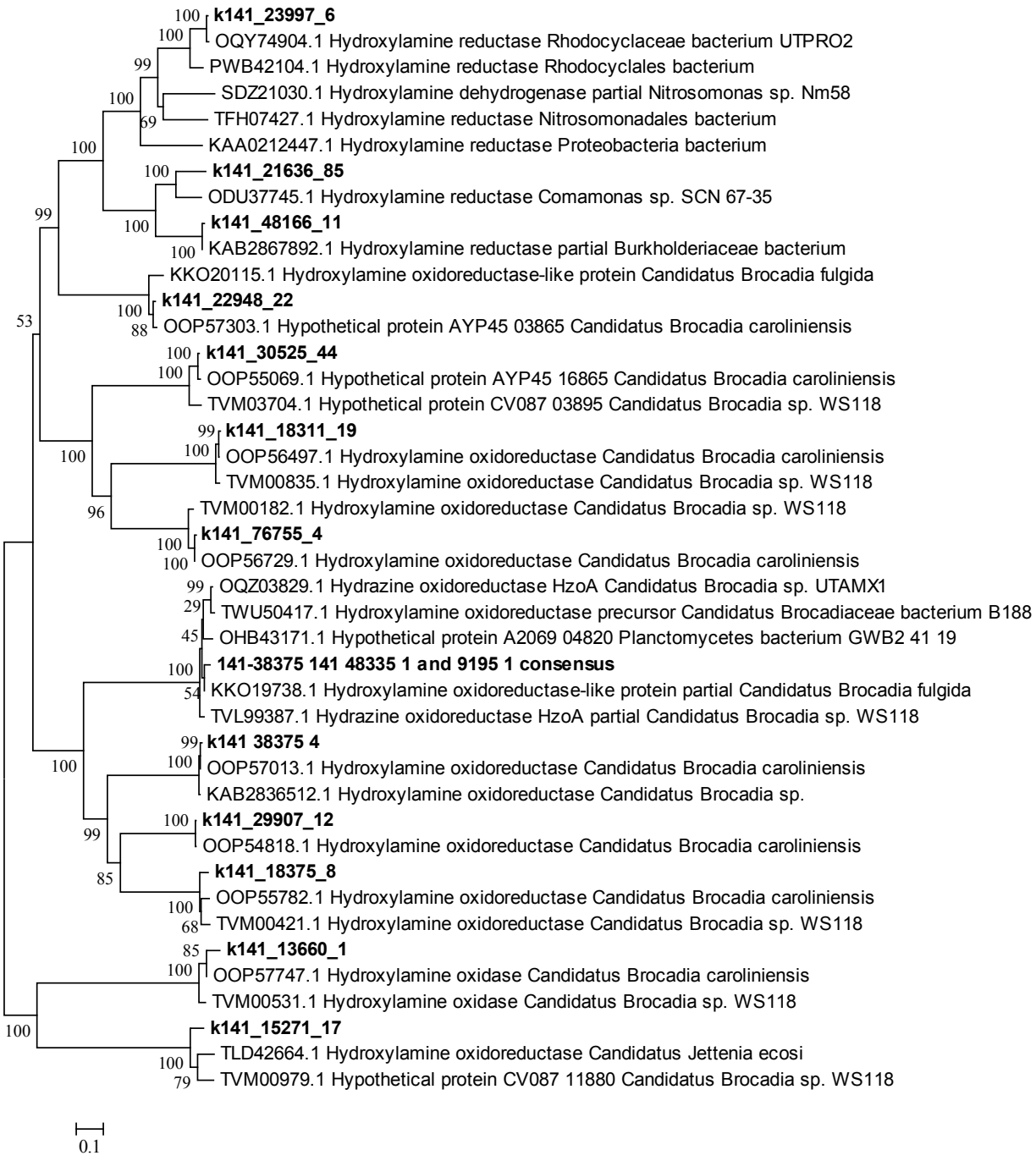

Figure 3.4.1 Phylogenic Tree of the Anammox Hzo Gene

Amino Acid Sequences of Hydroxylamine and Hydrazine Oxidoreductases. Full and partial length amino acid sequences related to hydroxylamine oxidoreductases and hydrazine oxidoreductases are listed below. 21 partial and full-length putative proteins are shown. 12 are full length (start and stop codons are present, and total length is about the expected length of the enzyme which is about 500-550 amino acids). 3 partial sequences (k141_38375_1, 48335_1, and 9195_1) had significant overall alignment (The last 47 AAs of 38375 had 100% identity to the first 47 AAs of 48335, the last 46 amino acids of which overlaps with the first 46 AAs in 9195_1). The consensus sequence appears to be a full-length protein. Additionally, k141_22377_1 overlaps with well over 170 AAs at the end, but has a different sequence along its terminal end for 20 AAs. Additionally, the partial sequence k141_40162_1 has 263 identical AAs from the start codon forward, except for the second AA in the sequence. It is unknown if this fragment represents a novel variation in a homologous gene or if it (or the consensus sequence) has 1 or more sequencing errors on the terminal end. Four partial sequences (k141_18447_1, k141_24469, k141_53470, k141_34078_1) have sequence KAA0212447 as their most identical sequence in pBLAST (shown in figure 3.4.1 above) but range in percent identity between 73% and 100% identical. No evidence could be found that these sequences have any identical overlap, so it is unknown how many fragments of genes they represent.

>k141_13660_1

MKKLFIVASCFVSLFMYPFASSAHQEGPGYIPSLFHEYTQMNLIYYTNYTSGIPDD
WLASRYSKTISDVDYIKPAFEGQVSEDTLQDTSRSALSEQTQVCLLCHKKSTPGI
VEDWLTSRHSKTTPDMALSKPVLERRISSDTIPENFRSVVVGCYECHSQNASAHK
DNFEHFGFRINVIVSPNDCRTCHSVEADQFAMSKKAHALDNLQKNPLYHTMVET
GLSSKGSSDGAIISITATENSRAESCYGCHGTEVTVNGMKKVSTPSGDIEVPNLTN
WPNQGVGRINPDGSSGACTACHPRHSFSIEIARKPYTCSQCHLEPDTPAFEVYEES
KHGNIFNSKQHEWNWNNVPWRIGKDFQAPTCAACHNSLITTSDGGVIAPRTHNF
GSRMWVRLFGLPYSHPQPKSGKTYEIKNKDEQPLPVTFTGELASEYLISKEEQMR
RQTEMKTICRSCHNTDWINGHFTKMDITIAETDKAVLAATQLLQKAWDKKLAD
PSNPFDEIIENDWVKQWLYYANSVRFGSAMGGYDYTTFKNGWLELTANLRKMQ
DFIETQK*

>k141_15271_17

MNNKTNWRKIFYLFLLFGLSVFDAVCAEAVEKQFPLDAKPGPVLTTANQMCVD
CHKTTTSALVMEWERSRHAQKGVGCIDCHKANEGEIDAWQHMGALISILVTPK
DCSNCHAIEAQEFSRSHHAKAGEILYSLDNLLAERVIGLPDNNADAVNGCLQCH
GSIIKFKRNDAGEILRDNGKPVLDPNTWPNSGIGRLNPDGSKGSCHACHSRHSFE
AKLSRAPENCGKCHMGPDHPQMEIYRESKHGIAYTANIDRMALDKEGRWVLGR
DYSAAPTCTTCHISNYMTSEGEHNASNHDVGERLSWTLRPVVSTKINTVIYEDGF
KEDYPDTRKLPEIGEKVQVIEKIVQDEKLVDKIVSKRVNKIVTWQERREKMKGV
CRNCHNHTHVDNFYQQFDSLVALYNKKFAEPAQAMMNALTEDKILNPNSPFEH
EVQWIFWELWHHEGRRARHGASMMGPDYTHWHGLYEVAKHYYMKFLPAVIK
VAARKSEEMKSKYEQKIEELLNQEEHLWIKGLSEEEINVLKSAYKNRYDE*

>k141_18311_19

MKLLRLGAILLAAPLVWSLQTGSLKAQSEAELKEMQKKATSYYDILYPNDTLKD
WFTTNVGLEKGAGPWQNIYKPVPLQMYWFPVRHYVKPDGTYYDQLLEKYKPS
DCVKCHEEVTPGFVNDWRDSTHANLRKNPRFAEKTQQIEKLIGRELKEVTCSDC
HGKDHKELHMPTPQVCGECHPQQTTEFMSEAERGRPNHIDGLAANVIPPWYPE
MFRRGYPAAQFGCDLCHSTDRCNICHTRHKFSAAEGRRPEACMSCHMGFDHPD
AETYGESKMGYIYHLEGEHWDWEKPLAEIVPGKDYRTPTCQFCHFDQGNGKFA
HNPVTKGVWRMGTVPPKGIDYKSSLKDYPHGINLPPLNYNLDIYSPENKKRSEQ
WVSVCSKCHSPRFARLYRENLNDFMFEMWRLQDRAQAILDDIVANDAFEVPIKE
RDIFPLGDILADALGPGLLGDAVYNAFKTKAGKVPVIGPILGLHGLFATGRNNPS
EIEITYADMWFGDKAHAYKGVAHGQQDIAWWYGAAKMYQKINILESQAIALKR
GKEVDKMLHAKGRKNVAGIVGSIVGIIAVIMFGAALWKKKRETQHS*

>k141_18375_8

MGKRYLLKQSFVNMLLFLSFIGVFIIPSLSTISFAIEPQDAGPQATETLIAKTTATPQ
RIFGNMRFRHEMADLLGEKVGPDDSGDVYNAGGLASYYTGPKELFPGKGKFGQ
LYKFLPIIRWYDPAYYFDAENFHPGSTVNGEFTNEQCITCHMLENPGIVTQWKQS
KHGTPSEGKEVVGCDRCHGKNHEKLLMPDYNLCGECHEKQLKGHRDGGRGSH
AHAYHLELIDQGCQMERPAEETTACLACHAIVENRCDGCHTRHRFSAAEARKPA
SCGVCHSGPEQYEYEMYMQSYHGMIYQGEGQTWDWTRRLNAKNYVVPTCAY
CHMPEGEHNVTRTSTVYTYMGTSLVDRGAYRYKATRDAWINMCKGCHSPRFA
KDHLEAMDEAVKLSFTKYREAMVIVTALYTDNLIDPMPADLAPDSQGHNVFSL
MPGKGEMRKYNVSNIERLTYEMLVDIVGAIYKAKAHNAYYSPIYGYWEWAQD
RWLVQIKDEATKLKRFAEIEEKLGIKHTAYSFWKHGEYTDMLLGWKRKEWGK*

>k141_18447_1 partial
LYDALGIRSDAPPKELYDALVKRYRDPAQGAGKGKFADLWQPIPFSKYFDPASF
YSPPSSVAEIAGRKDCVDCHEKESPGWVHQWRQSAHANLGKIRTLKPSDARYY
QKARLEEVERNLRSLGKLAANDSLKE

>k141_22377_1 partial
DLCPDWSGQHVWSLKIGAYHDDPAFGGKAGESGEFRMSNCSDIERLCFESVGYF
QTYIYKGMAHGSWNDATYSDGSFGMDRWLVNVKQDASQARRLAALEKKVGIT
WVPESFWKTGEWLDQLTGPYIVKNHPGKTIFDLCPDPGWLDTHHAPAEEVEYIN
RKIKELGWKTGHDAHHGDPHDEASRSMGKTH*

>k141_21636_85

MFARLLGLVTLALSLLVAHVPASAAIWDSVPDEQLKALGLTRDSSPKELFDRLSK
RYRSELTKGKFAKYWEPIPMDMYLAPTLFYKPPAEPAIEAKREECVACHTGATH
GWVKSWEKSVHANLDMIRALPESDSRAYKKAVIAEVEDNLRAQGLLRQGEPLK
EVQCIDCHMGVGAKSGNHAKDLRMPDRSVCGSCHVRQFAEAESERDTAVWPQ
KQWANGHPSHAVDYTANVETATWAALQQREVAASCTMCHTNQTKCDNCHTR
HEFSTVESRKPEACATCHNGVDHNEFEQYMYSKHGTVYQTLGAQWDWNIRLA
DQLGHKNGQTAPTCQSCHFEFKGQYSHNVVRKVRWGFLPSKDIADNIDHPWFK
DRKEAWVDTCSQCHSRRFAQEYLTMADKGIQDGSKLVEDTRKVVKKLYDDKL
LVGQKNNRPALPAPEQDEAGGFFSLFFAQANQTTTVDRTFAEMWEQHVARYMK
GLQHVNPGGWTYSHGWSDLVKDQAIINEQDTLLRERAAMEQRLQRLEGKGSGK
TSSYRSGERALYASVIEDPILASGGLALLGGGLLLTGLRGGRRRAAASADADTRT
QHDDVPG*

>k141_22948_22

MKVIWSFVGVAILFGVFCGGVPAQKLDKGVGPYGEFWKPIPTQRYWAPDYFYSP
PEEPKGVYQADECTLCHKALNPGLVKAWAESSHANLDKLQDYQKEKLAEIEKIL
GRKLTKVGCIDCHGKVGAEKLDHEKELIMPSSVLCGECHKQEYQEFESEKQYGIP
DWKPGRESHAKAYDANLDVDVWAAVDKNIVQGCDMCHSIQHKCDSCHTRHAF
KASEARRPEACQTCHNGPDHPDIEYYRDSKHGSIYFIEGHTWDWSKQLKDANYT
SPTCQSCHMYYKGQYSHNMVRKAIMGEGDVLFYDNVFKGIKPTDYIKNSKELM
ARREAWIEVCVQCHSPRFSRDYLASMDNASDSVFQYVSDAYATIKSLYEEGILYP
MPENRPKAPAPVTEKHPDLLGGFYGEFWAKNGNPSRIEKDFLYMWENDAFLVR
KGLAHMNPNGFTYLSWSNLLKKYVDIQSEANTLRRLAALEKKAKLRPRAKAKR
K*

>k141_23997_6

MIVKLWQKMLLLVCGILFAAGVHAEIPKETYDALKLDKSATSKQLYEALVKRY
KDPAEGAGRGTFAQYWEPIAFSKYLDPASSYKPPSSVKDVASRQQCVKCHADET
PVWVNTWKKSAHANLDNIRRLTPKDATFYKKAKLEDVEKNLRSLGKLAEGEQL
KEVGCIDCHVDVNTKKSADHRADLRMPTADVCATCHLQEFGERESERDTITWPK
DQWPKGRPSHALDFKANIETSIWAGMPQREIAEGCTMCHYNQNKCDGCHTRHE
FSVAESRKPQACATCHNGVDHNNWEAYSLSKHGKVVEIMGSKWNWDVPLKDA
FAKGGQSAPTCQSCHFEYQGKYSHNLVRKVRWANYPAAAGIAENIKSDWSERR
LEAWVKTCTNCHSESMARSYLDMMDKGTLQGLAKYQEAHKVVEKLYADKLLP
GQTTNRPTPPPTEKEGVAQFFQLFWTKGNNPSSVETEVIEMGENDLPKLHVGLA
HVNPGGFTYTEGWEPLSRAYSKIMSEDTKLREMAALQNRVAKLEAKRVSLLDS
DSNTGRASLGGLGGALLLAGGIALAGWRRRDRSGK*

>k141_24469_1 likely partial

MRKVRWANYPMVPGIAEAVVDSDWSRQRLGAWVKTCTQCHSERFARAYLEL
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MWSKGNNPTAVELEGKVLAENLLPKLFVGVAHVNPGGWTYTESWEQINAGYS
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GAALLWRSRRGRRDD*

>k141_29907_12

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ASYTGPEEVLPGEGKFGKLFGFLPLMRWYDPDHYYTPNMNVSGEFKHEECVMC
HTVQTPGIVAQWKKSKHSSTEKGVVGCDKCHGNNHQQLYMPSWKHCGKCHPE
QQAGHRAGKIGSHTHAFHVSVIEAPWQIAKPAAEVTACATCHGIAENRCDGCHT
RHDFSVAEARKPNNCGICHTGLDHYEYEMYKESYHGMIYESEQHTWDWTKPLK
PANYKTPTCAYCHMKDGEHNAQKASTINSHMGTSLVDRGAPKFKEARQNWINT
CKGCHSPRFAADQFEAMDEAVKVSFTKWREAMKIVVDLYNEGLLDPMPNDLAP
DYAGHYTFSLLGGEGRMYNVSDIERTSFEMLVYITNSVYKAMAHGAMYGATYG
KGAFLQDRWLIQIKGEASRLRRIKALENKVGIQHKAYDFWKHGEYTDLLLGWK
RKPGDVDKSKCMHEGENCLAE*

>k141_30525_44

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YHTHALEMGGDEGLSRGLWGALIIEPKDDITNPPDKEFVVFMHSYMVNGTEYEA
FNDKSGDIEFMNGDSSAFPGLVWKANMGEKVRFHVINTAEEMHTFHTHGHRWT
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VVEKEKMNAWMSPDTESLPTLDSAPGSYAYPDPSLKNLYEDFVGLKQGDGPWG
EFYQPIPFYMYFNPTRHYIPPSAENSDYKELLNRYRPDQCVECHEESSPGIVAQW
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KGIWRMGIIPPAEVEFKSGIKDFPYGIKLPPIDKKLEIYSAESKMKRRYWVELCTK
CHSSRFSSMWLDSLDQYMFESWRRIDEAQLVVEELFAKDMIRPSPDERAPFPLSD
VIVKLLSPKELGPEVYSLFKKTSGHLPVIGPVLGAYAIFNQKDGNPSGVEQEYVE
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AVKK*

>k141_34078_1 partial

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EFAERESERDTAVWPKGVERTKGRPSHALD

>k141_38375_1 partial

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PSNPIKGTFQLDQCIGCHSVITPGIVRDYKKSAHSRAEPSPTGCDTCHGNNHQKLT
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>k141_38375_4
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VFKNEECVLCHTVQTPTIVKDWKKSAHGNLEMRRGLGVKGKDGKPVEGTVGC
DICHGNDHQKLFMPTYKNCGECHPRETSEHRSGGLGSHTHAYTVNVLEFSWHV
GKPAEEVAGCAECHAIVENRCDGCHTRHVFSPAEARKPTACRYCHMGIDHDEW
AMYNTSVHGCLYESESATMDWSKPSKKGNYRVPTCAYCHMQDGNHNPQQYGT
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TKWREAAAVIVGCFLDGVVDPMPEGSPPDWYGHYTFSLLPGGDPRFYATSNLER
LGLEMICYLTGNVYKAYAHMSMYNQTYGNGSAFEQDRKLVEIKTEAAKLRRFA
AIEKKIGLEHKSEGFWQHGEYLDLLPGWIRKPGDVDVEWFKRTDIPHRANADAG
VAPHGH*

>k141_40162_1 partial

MGKFWKVTLASALISCGTMGVVANLMTKEVKAVEIITHWVPHEVYGQIGEPDN
NGKVFFSGLGAKYMGYPKDSGAPPYPGKYSKFWKTLPAYRYYIPDYMYNRDEV
RPSNPIKGTFQLDQCIGCHSVITPGIVRDYKKSAHSRAEPSPTGCDTCHGNNHQKL
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>k141_48166_11

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GWVQAWQQSVHANLDALRKLPDGDVRAYKKSILSEVEQGLRAQGVLAAGAPL
KEVGCIDCHMGVGKAGGNHARDLHLPSRADCGTCHIKQFAEAESERDTQVWPQ
KQWDPGHPSHAVDYTANVENTTWAALQQREVAASCTMCHYNQAKCDGCHTR
HDFSTVEARKPEACSTCHNGVDHNEFEQYMLSKHGTRYQTRGSTWDWNARLA
DAFTKGNQSAPTCQTCHMEFKGQYTHNVTRKVRWGFLPFTSIVDNLDHPWFKD
RKEAWVQTCSQCHSPRFATTYLNMSDSGIKEGVKLVESTRKVVQKLYDDKLLV
GQKTNRPAPPEPEKDEPGAFNSLFFSNGNNPTVVDRTFAEMWEQHIARYMKGLQ
HVNPGGWTYSHGWSDLIKDQTIINEYDTSLREKAALEQRVRKLEGTPKRRTSWI
GGAGAQQATLGLSVLDEQPLLASGALALLGVGLVFGGLRSGRRRAGSAGDDA*

>k141_48335_1 partial

TFCHTSPEERCSTCHQRHQFDPKVARKSEQCKTCHWGKDHRDWEAYDIGLHGT
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MSMADRGAPIWKEKRERWASVCDDCHSPRFAKENLQAMDESVKDAGLKYRET
FQIAADLVKDGVADPMPKDLCPDWSGQHVWSLKIGAYHDDPAFGGKAGESGEF
RMSNCSDIERL

>k141_53470_1 partial

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MEFNGKYGHNVVRKVRWANYPMVPGIAE

>k141_76755_4

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GVVRDWRGSGHKNPKSTPYLSARTSEIEKRTNRILNEVDCFDCHADTKKKQIRM
PTGEVCGECHRPQFDDFLREREMGRPNHIQSWEANTIVPWYAEAARRGYLYGQ
HGCDLCHSGAEKCDVCHTRHKFSAAEGRQPEACITCHMGPDHPDAESYGESKH
GVIYHKEEEHFDFNRPLSEVRPGKDYRTPTCQFCHMYEKHGRFIHNPVMKGIWR
MGTIPPKNLEYTSSLKDYPYGIKIIGDKIDIYSEENIAKRSYWLEVCAKCHSDRFA
DTYLKSLDEFMFQAHTLADRAQKIVEDLIADGYLYPSAADRDPYPLSDGIEKQLS
PAFLGEPIYNAFKTLKGKFPVVGPILGVYGMFIQQQDNPSNIENMYNRLWFWYK
LQGYKGTAHVQQDVSWWWGQAPMMMEFTKIQSEAARLRREGRIEKVALPK*

>k141_9195_1 partial

DLCPDWSGQHVWSLKIGAYHDDPAFGGKAGESGEFRMSNCSDIERLCFESVGYF
QTYIYKGMAHGSWNDATYSDGSFGMDRWLVNVKQDASQARRLAALEKKVGIT
WVPESFWKTGEWLDQLTGPYIVKNHPGKTIFDLCPDPGWLDTHHAPAEEVEYIN
RKIKELGWKTGFEHKLEHHEAGHDPAARSQRLSPHE*

Alignment of three partial sequences are shown in the following figures.

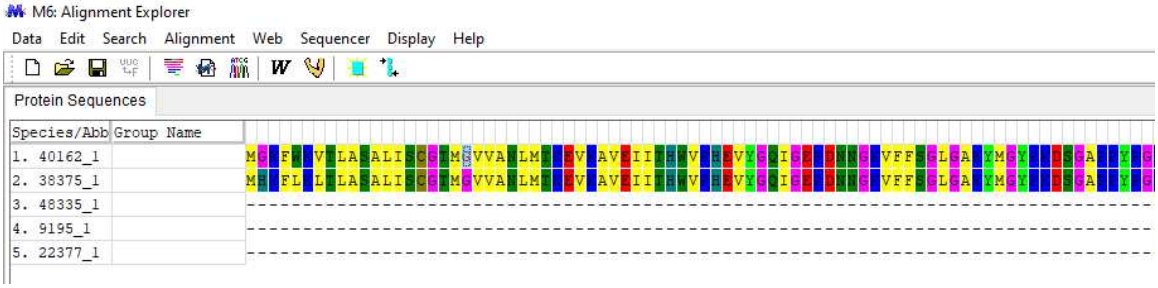

Figure 3.4.2

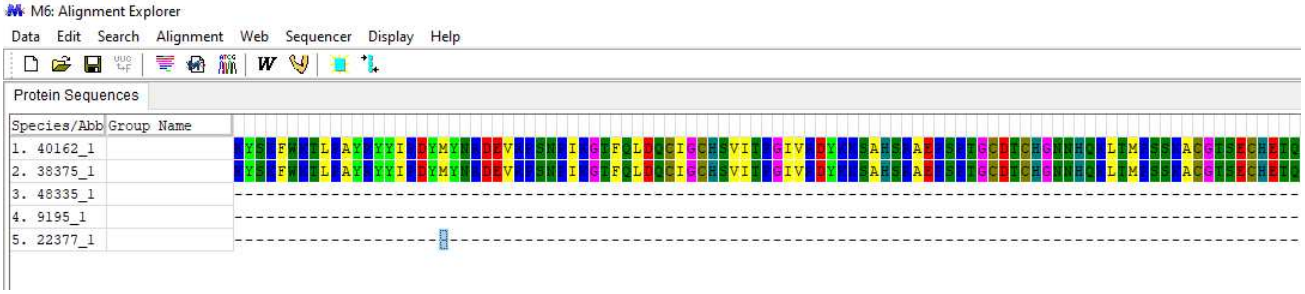

Figure 3.4.3

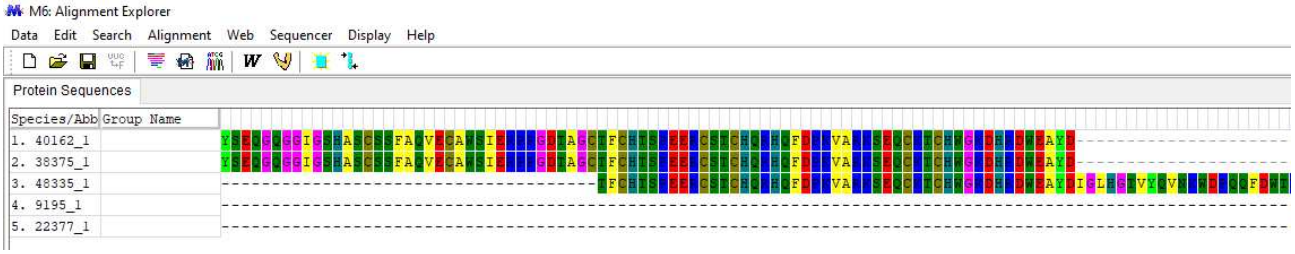

Figure 3.4.4

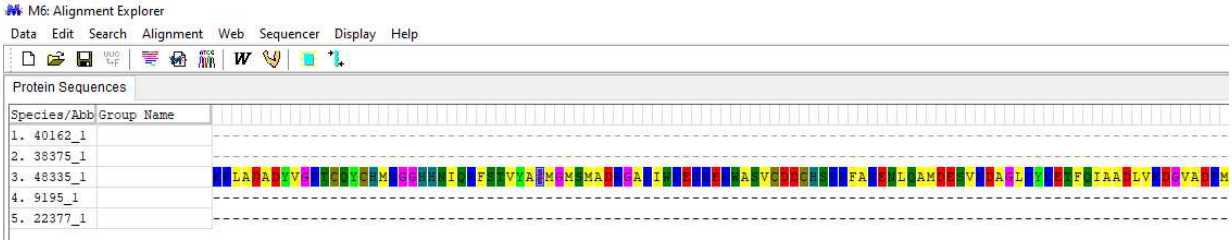

Figure 3.4.5

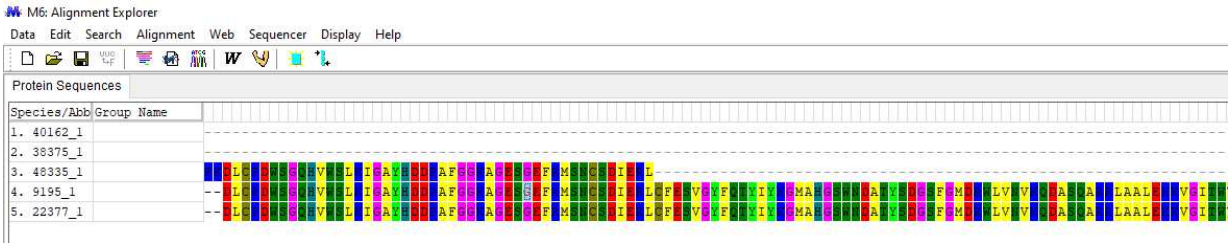

Figure 3.4.6

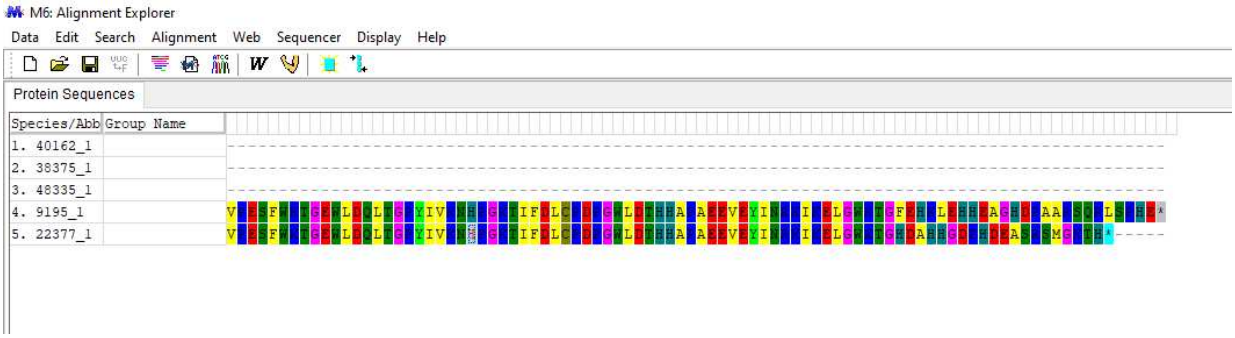

Figure 3.4.7

3.5 Metagenomic Data for Hydrazine Synthase Genes

Phylogenetic trees of Hydrazine Synthase genes, along with the amino acid sequences that were recovered in the dataset are shown below. There are three subunits of Hzs genes, each with their own tree in comparison of nearest matches in GenBank from pBlast.

Hydrazine Synthase Subunit A Phylogenic Tree

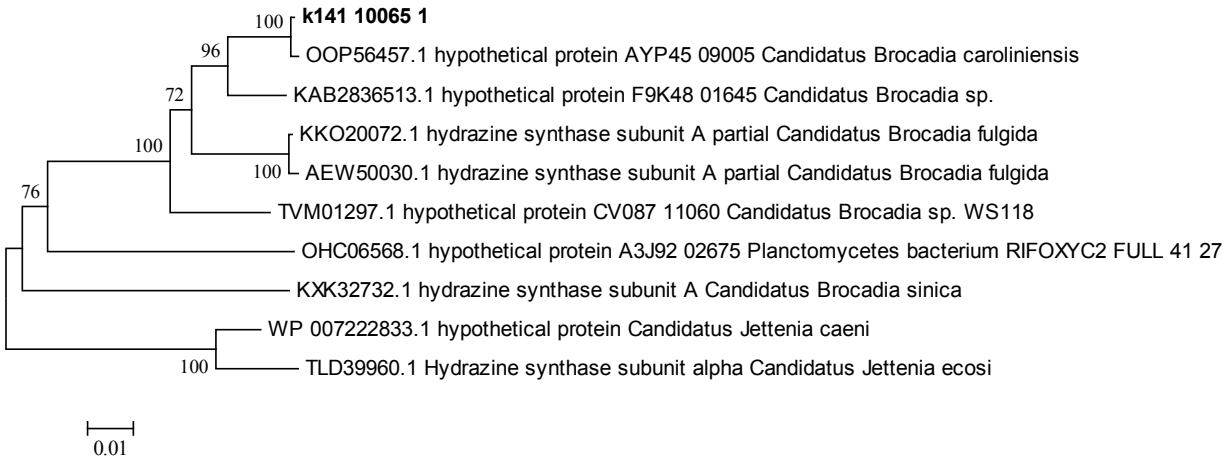

Figure 3.5.1 Phylogenic Tree of the Anammox Hzs Gene Sub A

#k141_10065_1 Hydrazine Synthase Subunit A

MSKRKIGGVVASAIMAGALVCGSVFASGNQVMVGGSKQGKALWTDYSGMSKEIQGPV
DAILFTQSPRTAKGDPYQNYPHYVPEGSRIVLYNLKTKELKVLTNDFATAFDPCTYWDG
KKFAFAGVHKKGGGCQIWEMNVDGSGLRQMTDYKGTCRAPIYYAAGSIEEGQGRIIWR
DRYFEGDWKERGTVEKTGFIIFAGSPDGVMDEFHNPYAYNLFRLDTQGGHVTERITGHV
LSGIEFPSINTTIDQITYNISSNFDPTTTPDGNILFSSTQANGSRAGGKGRVMLCVDNWDG
AYPRPIYGNCDNEIGGACGRSQAKISFGDRRLVFVESPYMNWGVGQLASVSWDAPYNK
TYERLTKDDGGLYRSPYPLPDDRMLVSYAERGDFGIYWFDCKNGKAGELVHNDPEWN
DHQPAPIYVKYRPRWINTFTAGKNFGVTTVTYQPFDQVKVEGYPHSWATWICFDTTLTD
LPVGPYPHQKSKDTKRGDVKAVRIVEGVPCVEPDANRFKAGAGSHLLGGCRSSSNSGTA
FQQRRIIGYQYVEDDGSVVTSQTADTPYYIQNLDERGMAVQTALMWAYLRPYHGRICS
GCHDGSYRGRAFQNQHTKALYNWWYDDRSHYDSPFAFGYLKFDRSGIYQGVKHGDDV
VVPSDVYYGGPSGTTSQPVEGLTDEKRRTVDFRRDLQPIIDVKCAGCHNGGTSPDLSGGS
ELVSVDGVAAFSRAYNTLLESQRGKDTNLGGKYVNPSSAINSLLIWRLYETALSQFSPRD
GVFPVEGRVMHDKFLTQDERYLFVEWIDIGAQWDNIQGPDLYPGVH*

Hydrazine Synthase Subunit B Phylogenic Tree

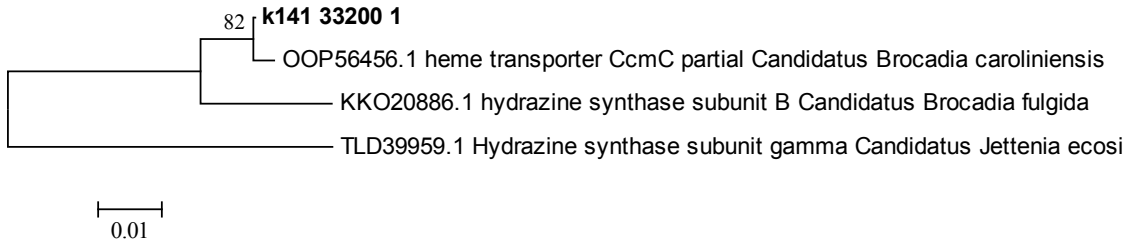

Figure 3.5.2 Phylogenic Tree of the Anammox Hzs Gene Subunit B

#k141_34637_1 Hydrazine Synthase Subunit B

MGAVLTVGGVASAGFIQGTHVKTDLPGPFHVTTSPDGGTLYICNQSGHSVTFVDARTMK
VTGEVAVGVQPEASAMTPDNAFLFVCNAESDSVSVIDIARKQVVKEIKVGDWPSGIRITK
DGRTAYVACSGCMWNTIDVIDTGKMEKTRSIYTSDYGPRTLDISPDGKTLAVINDTVGSI
NRSVNFIDVDSGRVTEKRIIRESANLRDVIYTPDGQYVVVTYETPKNWLPVCEAENGQVF
TNNIAVLETKAGGKVARLPLDELNNYDGNPYGLAMDPKGRYLYIGIRGMHRVTILDMG
KVLNVVRGNSQSDLDYLRDDLGFVREYLVARVTTGLGPSSVCLSPDGKYCYAANYFSN
NVSVIRTPVD*

Hydrazine Synthase Subunit C Phylogenic Tree

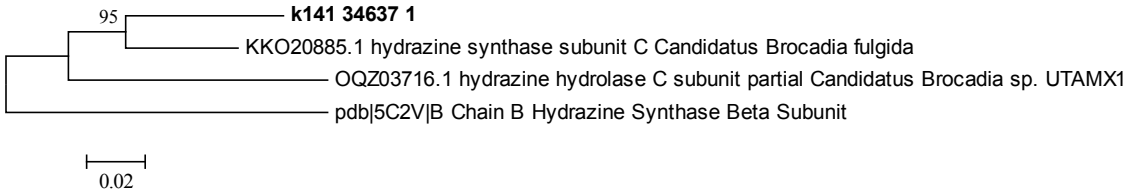

Figure 3.5.3 Phylogenic Tree of the Anammox Hzs Gene Subunit C

#k141_33200_1 1 729 1 Hydrazine Synthase Subunit C

MAGTPQVVATIQTGPEWEPLPRAEPLTVPEVHYRVKHSPYKSELVRYGQFQFNDASWSL
QGEYSCASCHYERGQTTGLIWDLGDEGWGSWKNTKYIRGGRYLPPFRHEGFTGHPDEIV
GATSSLDRVCGRDPGFVFRSENFSPLRLEALICYIRALEFTGSPFRNADGSLTDAQKRGEK
IFNDPNVGCAECHPGDVSDPKALFSDAQTHDVGTGRVGVKGFRSTPGKVFNLKALEAGE
DPYGEESDTPIIGLDLVKEFDTPTLRDIYASGTYFHDGSARLLIDTINNTVNDKDMHGRTS
HLTAQEMQDLVEFLKAL*

3.6 Quantification and Gel Electrophoresis Results for qPCR Standards

Standards were produced and tested with PCR to show dsDNA concentrations and band position.

Table 3.6.1 qPCR Standard Quantification

Primer Pair	Target	Gene bp	Concentration ng/μL	Reference
A438f/A684r	All anammox bacteria	248	17	Humbert
Amx368f/820r	*Ca. Kuenenia, Ca. Brocadia*	830	45	
HzoCl1f1/HzoCl1r2	Hydrazine dehydrogenase	470	54	Schmid 2008
Pla46f/1392r	Planctomycetes	1400	19	Schmid 2000

- Shown concentration are triplicate averages.

Gel electrophoresis showed that the lab made plasmid standards show expected band lengths and agrees that the standards created are amplified specifically by their respected primers. Figure 3.6.1 below shows the bp ladder, and all 4-primer pairs

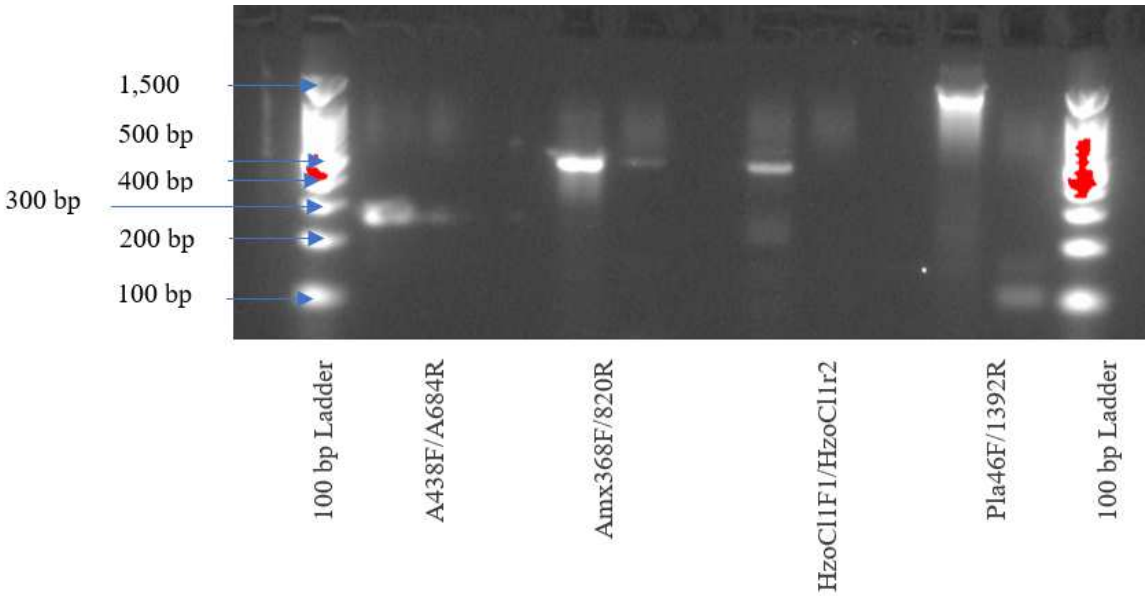

Figure 3.6.1 Gel Electrophoresis of qPCR Standards

3.7 qPCR Analysis

DNA extracts from reactors 1 and 2 from two time periods were analyzed using the plasmid standards to develop the standard curve. Standard curve analysis from qPCR shows the abundance of 16S rRNA for specific anammox bacteria and how these ratios change during mainstream applications. Table 3.7.1 shows the gene targets, efficiency, and R^2 values.

Table 3.7.1 Primer Efficiency and R^2 Values

Primer	Target	Efficiency	R^2
A438f/684r	All anammox bacteria	94.1%	0.994
Amx368f/820r	*Ca. Kuenenia, Ca. Brocadia*	93.1%	0.996
HzoCl1f1/1r2	Hydrazine dehydrogenase	95.1 %	0.995
Pla46f/1392r	Planctomycetes	79.5%	0.997

Using the qPCR standards, the abundance of each target gene is compared in reactors 1, 2, and the seed sludge from December and January. Figure 3.7.1 compares the log copy per ng of DNA of each target gene in different reactors.

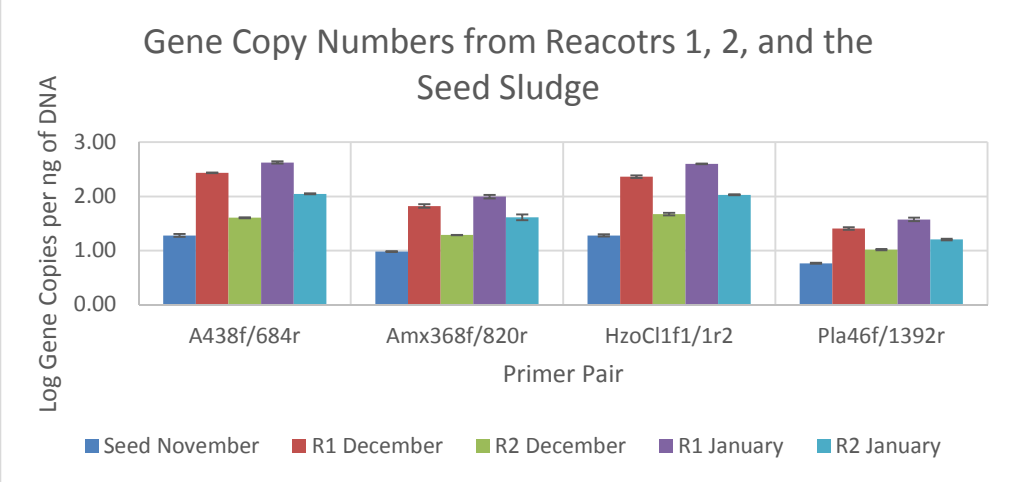

Figure 3.7.1 Log Copies of Genes per ng of DNA

CHAPTER IV

Discussion

4.1 Metagenomic Classification

There were 13 isolates from enriched anammox granules, and 77 complete isolates from single stage PN/A reactors 1, 2, and the seed sludge; all of which were derived from 16S rRNA sequencing. Of the 13 isolates in anammox granules, 2 were unknown families of the phylum *Chloroflexi* and 1 was an unknown family of the phylum *Firmicutes.* Furthermore, there were 5 phylums of *Chloroflexi*, 2 phylums of *Chlorobi*, and 1 phylum of *Planctomycetes*. These results agree with cited literature that anammox granules contain and abundance of species from the phylum *Chloroflexi*, which are thought to play important roles in the formation of granule structures (Christopher et al. 2017).

From the experimental PN/A reactors 1, 2, and the seed sludge; 77 complete genomes were found. These genomes consisted of 11 *Planctomycetes* and 16 *Chloroflexota*, and a diverse amount of 17 other phylum. Furthermore, there were 2 unknown families of the phylums *Chloroflexota*, *Actinobacteria*, and *Bdellovibrionota*

and 1 unknown family from the phylums *Hydrogenedentota*, *Fen-1099*, *Myxococcota*, and *Verrucomicrobiota.*

Furthermore, there were 7 genomes from *Planctomycetes Phycisphaerae* and 11 *Chloroflexota Anaerolineae* which could contribute to anammox granules (Zehand et. al. 2017). An interesting finding from the experimental PN/A reactor is a complete genome that is classified into a completely unknown phylum. Bin 133 contains a genome that is 87.91% complete with only 1.1% contamination (figure 3.3.1). This unknown culture requires much more analysis to determine its roll, if any, in PN/A reactors.

Unsurprisingly, the only anammox genome recovered from both data sets was very closely related to other *Brocadia caroliniensis* (genome designation: K141_59870). However, it was also expected to potentially find *Brocadia fulgida*, since this species has been shown to dominate anammox wastewater cultures in some previous studies (Kartal et al. 2008; Gori et al. 2011).

4.2 Metagenomic Discussion from Hydrazine Oxidase Genes

From the granule metagenome, the recovered Hzo and hydroxylamine gene sequence alignment was done using MEGA6. The metagenomic bioinformatics and blast logarithm did not produce a fully complete Hzo gene, but rather recalled three partial genes that appear to have significant overlap. Recognizing this, the sequences were aligned manually to produce a complete ‘consensus’ sequence. The resulting alignment shows that the sequences K141_38375, K141_48335 1, K141_9195 1 were all partial sequences of the anammox Hzo gene and had significant overall alignment. The last 47 AAs of 38375 had 100% identity to the first 47 AAs of 48335, the last 46 amino acids of

which overlaps with the first 46 AAs in 9195_1. This consensus sequence appears to be a full-length protein. Additionally, k141_22377_1 overlaps with well over 170 AAs at the end, but has a different sequence along its terminal end for 20 AAs. Additionally, the partial sequence k141_40162_1 has 263 identical AAs from the start codon forward, except for the second AA in the sequence. It is unknown if this fragment represents a novel variation in a homologous gene or if it, or the consensus sequence, has 1 or more sequencing errors on the terminal end. Regardless, this consensus protein sequence will serve to make new biomarkers for future studies of anammox bacteria.

4.3 Metagenomic Discussion for the Hydrazine Synthase Genes

Three subunits of the Hzs gene were identified from metagenomic data. For the Hzs subunit A, the closet protein to the genome designation K141_10651 was the hypothetical protein AYP45 09005 from *Brocadia caroliniensis.* For the Hzs subunit B, the genome designation K141_332001 was most closely related to a partial sequence of *Brocadia caroliniensis* heme transporter Ccme gene. For the Hzs subunit C, the genome designation K141_346371 was most closely related to *Brocadia fulgida* but the sequence identity shows to have a greater deal of divergence (figure 3.5.3). *Brocadia fulgida* has been previously shown to oxidize acetate at the highest rate and dominate the anammox community in anammox enrichment cultures in the presence of acetate (Kartal et al. 2008; Gori et al. 2011).

4.4 qPCR Discussion

Data from qPCR methods on DNA extracts from PN/A reactors show high R^2 values ($R^2 > 0.994$). The efficiency of the qPCR protocols was 94.1%, 93.1%, 95.1%, and 79.5% for primer pairs A438f/A684r, Amx368f/Amx820r, HzoCl1f1/HzoCl1r2, and Pla46f/1392r respectively. Analyzing qPCR results show that all 16S rRNA genes from anammox bacteria were 2.67 and 1.77 log copies per ng of DNA in reactors 1 and 2 respectively (during stress inducing conditions in reactor 2). These same two reactors show that Ca. *Kuenenia* and *Ca. Brocadia* 16S rRNA genes had an abundance of 1.82 and 1.29 log copies of 16S rRNA per ng of DNA in reactors 1 and 2 respectively. These results are less than other reported qPCR results in Wang et. al. 2016; Moore et. al. 2011; Sonthiphand and Neufeld 2013, Which report an average of 7.7 log copies per mL of DNA extract, 7.7 copies per ng of DNA, and 4 log copies per ng of DNA respectively. Further analysis show that the abundance of the phylum *Planctomycetes* 16S rRNA copies were 1.64 and 1.18 log copies per ng DNA in reactors 1 and 2 respectively. These results are not surprising as the abundance of *Planctomycete*s is positively correlated to the higher abundance of anammox bacteria. However, these results seem to suggest that anammox bacteria contain more than the average copy number of 16S rRNA per cell than all other bacteria. Previous studies state that the average number of 16S rRNA per cell in all bacteria were 3.6 copies per cell (Harm et. al. 2003). However, the copies per anammox cell are uncertain. This may explain why there are more log copies of 16S rRNA per ng of DNA in all anammox bacteria, as well as *Ca. Kuenenia* and *Ca. Brocadia,* than there are for all *planctomycetes* bacteria present in the DNA extractions. Another possible explanation is that the universal primer pair targeting all

Planctomycetes (Pla46f/1392r) bacteria has a greater degree of selectivity than previously thought. This is visualized in figure 4.4.1

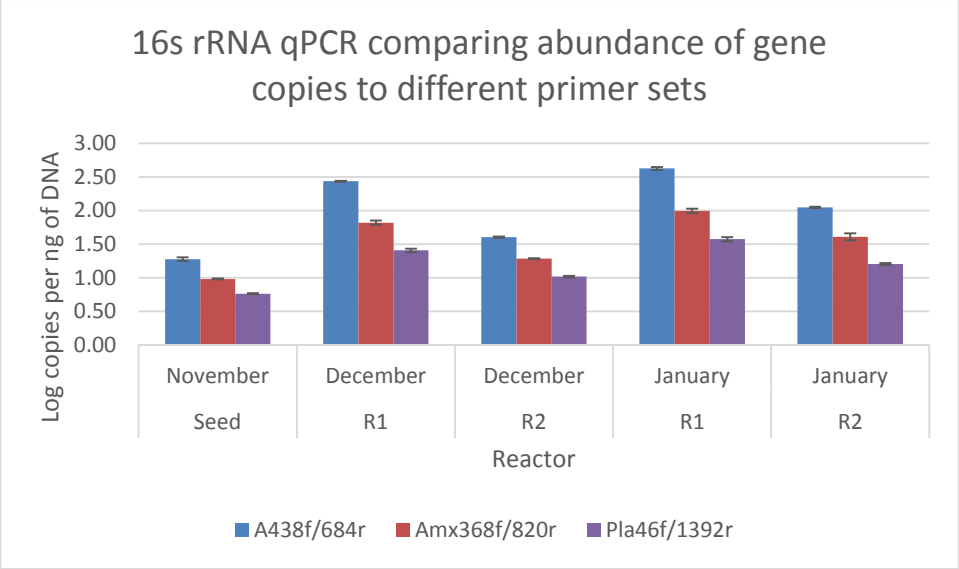

Figure 4.4.1 Abundance of Different Anammox Bacteria

This figure (figure 4.4.1) shows that there were always more copies of 16S rRNA copies per ng of DNA in all anammox bacteria than for all *Planctomycetes* bacteria.

Analyzing the Hzo gene with the primer set HzoCl1f1/HzoCl1r2 yielded 2.36 and 1.67 log copies per ng of DNA in reactors 1 and 2 respectively. This data follows the trend that there were more copies of anammox genes in reactors 1 than there were in reactor 2. This phenomenon is explained by the greater abundance of anammox bacteria present in un-stressed ANAMMOX reactors.

CHAPTER V

Conclusion

Characterizing the microbial community that makes up highly enriched anammox granules and PN/A reactors is a tremendous step toward furthering the scientific understanding of how complex microbial interactions work together to benefit the environment. No single bacteria to date can degrade the pollutants as a whole; and they must incorporate synergistic relationships in order to grow. Understanding these relationships among microbial communities is the corner stone to applying biological pollutant removal to any system.

From metagenomic analysis and 16S rRNA sequencing, it was found that only one species of anammox bacteria was present in enriched anammox granules as well as in biofilms recovered off of plastic media in single stage PN/A reactors. *Brocadia caroliniensis* was the sole member of the anammox family to be present in all ANAMMOX reactors. It was also found that the class *Anaerolineae* of the *Chloroflexi* phylum was a dominate group of bacteria found in enriched anammox granules. In comparison, the biological community made up of enriched granules derived from granule DNA extract revealed 13 complete genomes whereas DNA extracts from PN/A reactors uncovered 77 genomes, indicating that biofilm grown on plastic media in PN/A reactors support a more diverse community than anammox granules alone. Many

members of the PN/A reactors are nitrifying bacteria, which are needed to oxidize ammonia and supply nitrite to the ANAMMOX bacteria.

Protein sequencing data provided 24 partial and complete genomes, three of which have been compiled to form a consensus Hzo protein closely homologous to an Hzo from a *Brocadia caroliniensis.* The consensus protein is a full-length protein that will serve as a template to create new biomarkers in future anammox studies.

From qPCR analysis, the abundance of *C. Brocadia* and *C. Kuenenia* as well as all other anammox bacteria and all members of *Planctomycetes* were lower after a month in acetate stressed conditions. This trend is also true for the Hzo protein. However, the abundance of *C. Brocaida* and *C. Kuenenia* are greater than all *Planctomycetes*. This could be considered an anomaly of the data set due to a greater selectivity of the universal primer set Pla46f/1392r than originally expected.

Anammox bacteria have a key role in better processing nutrient nitrogen while forwarding cost of increasing nitrogen limits in WWTPs. By reducing the amount of required oxygen, ANAMMOX can greatly benefit WWTPs while simultaneously producing resources that WWTPs can use. This study shows that key functional genes can be analyzed to find improved primer sets so that enriched anammox granules can be used in the mainstream waste flow of WWTPs in large scale applications. Understanding anammox bacterial stress response will allow for a greater spectrum of ANAMMOX applications in water treatment.

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APPENDICES

qPCR Data for A438f/684r.

Well	Fluor	Target	Content	Sample	Cq	SQ			log copies per std	average Cq	average log copy
A01	SYBR	A438f/684r	Std-1		21.57	1,000,000		3.53E+08	8.55	22.03	8.55
A02	SYBR	A438f/684r	Std-1		22.25	1,000,000			8.55		
A03	SYBR	A438f/684r	Std-1		22.28	1,000,000			8.55		
A04	SYBR	A438f/684r	Std-2		25.53	100,000			7.55	25.61	7.53
A05	SYBR	A438f/684r	Std-2		25.63	100,000			7.55		
A06	SYBR	A438f/684r	Std-2		25.67	100,000			7.55		
A07	SYBR	A438f/684r	Std-3		29.00	10,000			6.55	28.98	6.56
A08	SYBR	A438f/684r	Std-3		28.77	10,000			6.55		
A09	SYBR	A438f/684r	Std-3		29.17	10,000			6.55		
								log copies/ng DNA	stdev	% dev	avg log copy
C01	SYBR	A438f/684r	Unkn		15.21	94,529,557	10.51	2.44	0.0064	0.26%	2.44
C02	SYBR	A438f/684r	Unkn		15.44	80,865,268	10.44	2.43			
C03	SYBR	A438f/684r	Unkn		15.34	86,571,213	10.47	2.43			
C04	SYBR	A438f/684r	Unkn		16.97	29,408,644	10.00	1.61	0.0088	0.55%	1.61
C05	SYBR	A438f/684r	Unkn		17.41	21,963,332	9.88	1.59			
C06	SYBR	A438f/684r	Unkn		17.05	27,783,294	9.98	1.61			
C07	SYBR	A438f/684r	Unkn		15.81	63,107,505	10.33	2.65	0.0216	0.82%	2.63
C08	SYBR	A438f/684r	Unkn		16.04	54,208,313	10.27	2.63			
C09	SYBR	A438f/684r	Unkn		16.52	39,475,521	10.13	2.60			
C10	SYBR	A438f/684r	Unkn		17.61	19,138,381	9.82	2.05	0.0086	0.42%	2.05
C11	SYBR	A438f/684r	Unkn		17.75	17,442,370	9.78	2.04			
C12	SYBR	A438f/684r	Unkn		17.40	22,034,276	9.88	2.06			
D01	SYBR	A438f/684r	Unkn		15.66	69,971,481	10.38	1.27	0.0267	2.09%	1.28
D02	SYBR	A438f/684r	Unkn		16.04	54,183,842	10.27	1.25			
D03	SYBR	A438f/684r	Unkn		14.26	176,574,467	10.78	1.31			

qPCR Data for AMX368f/820r

Well	Fluor	Target	Content	Sample	Cq	SQ			log copies per std	average Cq	average log copy
A04	SYBR	Amx368f/820r	Std-2		11.54	100,000	5	5.00E+08	8.70	11.50	8.63
A05	SYBR	Amx368f/820r	Std-2		11.82	100,000	5		8.70		
A06	SYBR	Amx368f/820r	Std-2		11.13	100,000	5		8.70		
A07	SYBR	Amx368f/820r	Std-3		14.40	10,000	4		7.70	14.60	7.74
A08	SYBR	Amx368f/820r	Std-3		13.99	10,000	4		7.70		
A09	SYBR	Amx368f/820r	Std-3		15.41	10,000	4		7.70		
A10	SYBR	Amx368f/820r	Std-4		17.85	1,000	3		6.70	17.93	6.80
A11	SYBR	Amx368f/820r	Std-4		18.02	1,000	3		6.70		
A12	SYBR	Amx368f/820r	Std-4		17.92	1,000	3		6.70		
B01	SYBR	Amx368f/820r	Std-5		21.81	100	2		5.70	21.98	5.64
B02	SYBR	Amx368f/820r	Std-5		21.68	100	2		5.70		
B03	SYBR	Amx368f/820r	Std-5		22.45	100	2		5.70		
B04	SYBR	Amx368f/820r	Std-6		25.37	10	1		4.70	25.55	4.63
B05	SYBR	Amx368f/820r	Std-6		25.69	10	1		4.70		
B06	SYBR	Amx368f/820r	Std-6		25.58	10	1		4.70		
B07	SYBR	Amx368f/820r	Std-7		28.51	1	0		3.70	28.60	3.76
B08	SYBR	Amx368f/820r	Std-7		28.76	1	0		3.70		
B09	SYBR	Amx368f/820r	Std-7		28.53	1	0		3.70		
								log copies/ng DNA	stdev	% dev	
C01	SYBR	Amx368f/820r	Unkn		13.86	18,325	7.95	1.85	0.0322	1.8%	1.82
C02	SYBR	Amx368f/820r	Unkn		14.22	14,483	7.85	1.83			
C03	SYBR	Amx368f/820r	Unkn		14.82	9,724	7.68	1.79			
C04	SYBR	Amx368f/820r	Unkn		13.91	17,673	7.94	1.28	0.0050	0.4%	1.29
C05	SYBR	Amx368f/820r	Unkn		13.72	20,119	7.99	1.29			
C06	SYBR	Amx368f/820r	Unkn		13.73	19,897	7.99	1.29			
C07	SYBR	Amx368f/820r	Unkn		13.94	17,400	7.93	2.03	0.0334	1.7%	2.00
C08	SYBR	Amx368f/820r	Unkn		14.64	10,991	7.73	1.98			
C09	SYBR	Amx368f/820r	Unkn		14.80	9,878	7.69	1.97			
C10	SYBR	Amx368f/820r	Unkn		14.37	13,098	7.81	1.63	0.0531	3.3%	1.61
C11	SYBR	Amx368f/820r	Unkn		13.92	17,649	7.94	1.65			
C12	SYBR	Amx368f/820r	Unkn		15.64	5,662	7.45	1.55			
D01	SYBR	Amx368f/820r	Unkn		13.42	24,525	8.08	0.99	0.0074	0.8%	0.98
D02	SYBR	Amx368f/820r	Unkn		13.77	19,471	7.98	0.97			
D03	SYBR	Amx368f/820r	Unkn		13.38	25,152	8.09	0.99			

qPCR Data for HzoCl1f1/Hzocl1r2

Well	Fluor	Target	Content	Sample	Cq	SQ		log copies per std	average Cq	average log copy	
A01	SYBR	Hzo	Std-1		18.29	1,000,000	1.06E+09	9.02719541	18.06	9.156645717	
A02	SYBR	Hzo	Std-1		17.84	1,000,000		9.02719541			
A04	SYBR	Hzo	Std-2		21.48	100,000		9.02719541	21.63	8.226418714	
A05	SYBR	Hzo	Std-2		21.79	100,000		8.02719541			
A06	SYBR	Hzo	Std-2		21.63	100,000		8.02719541			
A07	SYBR	Hzo	Std-3		25.12	10,000		8.02719541	25.16	7.306320825	
A08	SYBR	Hzo	Std-3		25.25	10,000		7.02719541			
A09	SYBR	Hzo	Std-3		25.13	10,000		7.02719541			
A10	SYBR	Hzo	Std-4		29.00	1,000		7.02719541	28.40	6.462546481	
A11	SYBR	Hzo	Std-4		28.10	1,000		6.02719541			
A12	SYBR	Hzo	Std-4		28.11	1,000		6.02719541			
								log copies/ng	stdev	% dev	avg log copy
C01	SYBR	Hzo	Unkn		14.81	9,366,733	10.005	2.33	0.0264	1.12%	2.36
C02	SYBR	Hzo	Unkn		13.98	16,259,533	10.220	2.38			
C03	SYBR	Hzo	Unkn		13.81	18,232,354	10.265	2.39			
C04	SYBR	Hzo	Unkn		14.10	15,012,307	10.189	1.64	0.0254	1.52%	1.67
C05	SYBR	Hzo	Unkn		13.53	21,948,320	10.337	1.67			
C06	SYBR	Hzo	Unkn		12.63	40,050,531	10.572	1.71			
C07	SYBR	Hzo	Unkn		14.36	12,630,939	10.122	2.60	0.0039	0.15%	2.60
C08	SYBR	Hzo	Unkn		14.22	13,868,666	10.158	2.60			
C09	SYBR	Hzo	Unkn		14.31	13,050,074	10.134	2.60			
C10	SYBR	Hzo	Unkn		16.00	4,213,725	9.694	2.02	0.0096	0.47%	2.03
C11	SYBR	Hzo	Unkn		15.58	5,595,568	9.804	2.04			
C12	SYBR	Hzo	Unkn		15.87	4,609,892	9.729	2.03			
D01	SYBR	Hzo	Unkn		12.30	50,169,830	10.660	1.30	0.0207	1.62%	1.28
D02	SYBR	Hzo	Unkn		13.84	17,880,128	10.257	1.25			
D03	SYBR	Hzo	Unkn		12.73	37,591,276	10.547	1.29			

qPCR Data for Pla46f/1392r

Well	Fluor	Target	Content	Sample	Cq	SQ		log copies per std	average Cq	average log copy	
A01	SYBR	Pla46f/1392r	Std-1		6.75	1,000,000	1.26E+08	8.10	7.01	7.95	
A02	SYBR	Pla46f/1392r	Std-1		7.05	1,000,000		8.10			
A03	SYBR	Pla46f/1392r	Std-1		7.24	1,000,000		8.10			
A04	SYBR	Pla46f/1392r	Std-2		9.95	100,000		7.10	9.99	7.20	
A05	SYBR	Pla46f/1392r	Std-2		10.07	100,000		7.10			
A06	SYBR	Pla46f/1392r	Std-2		9.95	100,000		7.10			
A07	SYBR	Pla46f/1392r	Std-3		13.76	10,000		6.10	14.03	6.17	
A08	SYBR	Pla46f/1392r	Std-3		14.06	10,000		6.10			
A09	SYBR	Pla46f/1392r	Std-3		14.27	10,000		6.10			
A10	SYBR	Pla46f/1392r	Std-4		17.89	1,000		5.10	18.12	5.14	
A11	SYBR	Pla46f/1392r	Std-4		17.87	1,000		5.10			
A12	SYBR	Pla46f/1392r	Std-4		18.60	1,000		5.10			
B01	SYBR	Pla46f/1392r	Std-5		22.52	100		4.10	22.27	4.09	
B02	SYBR	Pla46f/1392r	Std-5		22.17	100		4.10			
B03	SYBR	Pla46f/1392r	Std-5		22.11	100		4.10			
B04	SYBR	Pla46f/1392r	Std-6		26.33	10		3.10	26.38	3.05	
B05	SYBR	Pla46f/1392r	Std-6		26.49	10		3.10			
B06	SYBR	Pla46f/1392r	Std-6		26.31	10		3.10			
								log copies/ng	stdev	% dev	avg log copy
C01	SYBR	Pla46f/1392r	Unkn		13.98	12,262	6.19	1.44	0.0246	1.75%	1.41
C02	SYBR	Pla46f/1392r	Unkn		15.00	6,754	5.93	1.38			
C03	SYBR	Pla46f/1392r	Unkn		14.57	8,692	6.04	1.40			
C04	SYBR	Pla46f/1392r	Unkn		13.71	14,414	6.26	1.01	0.0121	1.19%	1.02
C05	SYBR	Pla46f/1392r	Unkn		13.09	20,642	6.41	1.03			
C06	SYBR	Pla46f/1392r	Unkn		13.73	14,195	6.25	1.01			
C07	SYBR	Pla46f/1392r	Unkn		13.51	16,144	6.31	1.62	0.0326	2.07%	1.57
C08	SYBR	Pla46f/1392r	Unkn		14.26	10,410	6.12	1.57			
C09	SYBR	Pla46f/1392r	Unkn		14.73	7,907	6.00	1.54			
C10	SYBR	Pla46f/1392r	Unkn		15.76	4,331	5.74	1.19	0.0142	1.18%	1.20
C11	SYBR	Pla46f/1392r	Unkn		15.78	4,281	5.73	1.19			
C12	SYBR	Pla46f/1392r	Unkn		15.20	6,006	5.88	1.22			
D01	SYBR	Pla46f/1392r	Unkn		13.97	12,338	6.19	0.75	0.0084	1.10%	0.76
D02	SYBR	Pla46f/1392r	Unkn		13.31	18,133	6.36	0.78			
D03	SYBR	Pla46f/1392r	Unkn		13.72	14,288	6.25	0.76			

Metagenomic Data from PN/A Reactors and the Seed Sludge

Bin	classification
bin. 176	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagaceae;g__Ferruginibacter;s__Ferruginibacter sp001898465
bin. 206	d__Bacteria;p__Actinobacteriota;c__Acidimicrobiia;o__IMCC26256;f__;g__;s__
bin. 40	d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__UBA2796;g__UBA2796;s__UBA2796 sp002352035
bin. 123	d__Bacteria;p__Hydrogenedentota;c__Hydrogenedentia;o__Hydrogenedentiales;f__;g__;s__
bin. 168	d__Bacteria;p__OLB16;c__OLB16;o__OLB16;f__OLB16;g__;s__
bin. 129	d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__Phycisphaerales;f__SM1A02;g__UBA2396;s__
bin. 118	d__Bacteria;p__Actinobacteriota;c__Acidimicrobiia;o__UBA2766;f__;g__;s__
bin. 94	d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__UBA2029;g__;s__
bin. 112	d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__UBA1845;f__UBA1845;g__;s__
bin. 72	d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__UBA1845;f__Fen-1342;g__;s__
bin. 71	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Comamonas_D;s__
bin. 96	d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__UBA2029;g__;s__
bin. 83	d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Dongiales;f__Dongiaceae;g__;s__
bin. 70	d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__;s__
bin. 85	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__PHOS-HE28;g__PHOS-HE28;s__
bin. 19	d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Bryobacterales;f__Bryobacteraceae;g__;s__
bin. 81	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Comamonas_D;s__Comamonas_D granuli
bin. 162	d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__Phycisphaerales;f__SM1A02;g__;s__
bin. 5	d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__UBA1845;f__Fen-1342;g__;s__
bin. 82	d__Bacteria;p__Planctomycetota;c__UBA8742;o__UBA2392;f__UBA2392;g__UBA2392;s__UBA2392 sp002343805
bin. 51	d__Bacteria;p__Bacteroidota;c__Kapabacteria;o__Kapabacteriales;f__Kapabacteriaceae;g__OLB6;s__OLB6 sp001567175
bin. 127	d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Pedosphaerales;f__Pedosphaeraceae;g__;s__
bin. 207	d__Bacteria;p__FEN-1099;c__FEN-1099;o__;f__;g__;s__

bin.92	*d__Bacteria;p__Bdellovibrionota;c__Bacteriovoracia;o__;f__;g__;s__*
bin.111	*d__Bacteria;p__Planctomycetota;c__Brocadiae;o__Brocadiales;f__Brocadiaceae;g__Brocadia;s__Brocadia caroliniensis*
bin.171	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Ferrovibrionales;f__Ferrovibrionaceae;g__;s__*
bin.33	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__AKYH767-A;f__OLB10;g__OLB10;s__OLB10 sp001567275*
bin.181	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Ga0077523;g__;s__*
bin.188	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Anaerolineales;f__envOPS12;g__OLB14;s__*
bin.57	*d__Bacteria;p__Myxococcota;c__Polyangia;o__Polyangiales;f__Polyangiaceae;g__;s__*
bin.28	*d__Bacteria;p__Bdellovibrionota;c__Bacteriovoracia;o__;f__;g__;s__*
bin.150	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__A4b;g__OLB15;s__*
bin.45	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__4572-78;f__;g__;s__*
bin.21	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagaceae;g__UBA1930;s__*
bin.64	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__koll-22;g__;s__*
bin.87	*d__Bacteria;p__GWC2-55-46;c__GWC2-55-46;o__GWC2-55-46;f__GWC2-55-46;g__UBA5799;s__*
bin.60	*d__Bacteria;p__Spirochaetota;c__Leptospirae;o__Leptospirales;f__Leptonemataceae;g__Leptonema;s__Leptonema illini*
bin.113	*d__Bacteria;p__Myxococcota;c__Myxococcia;o__Myxococcales;f__Myxococcaceae;g__;s__*
bin.158	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Dongiales;f__Dongiaceae;g__;s__*
bin.140	*d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Bryobacterales;f__;g__;s__*
bin.6	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Anaerolineales;f__envOPS12;g__UBA12294;s__*
bin.73	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__A4b;g__GCA-2702065;s__*
bin.179	*d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Bryobacterales;f__Bryobacteraceae;g__UBA690;s__UBA690 sp003487005*
bin.66	*d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Opitutales;f__Opitutaceae;g__UBA2377;s__*
bin.166	*d__Bacteria;p__Myxococcota;c__UBA9042;o__UBA9042;f__;g__;s__*
bin.68	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__SCN-69-89;s__*
bin.147	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__UBA2796;g__;s__*
bin.157	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__A4b;g__;s__*

bin.56	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__Azospira;s__Azospira oryzae*
bin.131	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__UBA4142;f__UBA4142;g__;s__*
bin.137	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__A4b;g__OLB13;s__OLB13 sp001567485*
bin.187	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Nitrosomonadaceae;g__Nitrosomonas;s__*
bin.193	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__UBA7966;f__UBA7966;g__;s__*
bin.37	*d__Bacteria;p__Chloroflexota;c__Dehalococcoidia;o__UBA2991;f__UBA2991;g__;s__*
bin.13	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Dongiales;f__Dongiaceae;g__;s__*
bin.75	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;g__Pseudorhodoplanes;s__*
bin.116	*d__Bacteria;p__Desulfobacterota_A;c__Desulfovibrionia;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio_A;s__*
bin.143	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Rhodanobacteraceae;g__Dokdonella;s__*
bin.26	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Anaerolineales;f__;g__;s__*
bin.148	*d__Bacteria;p__Bacteroidota;c__Ignavibacteria;o__SJA-28;f__B-1AR;g__;s__*
bin.108	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__PHOS-HE28;g__PHOS-HE28;s__*
bin.132	*d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__UBA1845;f__Fen-1342;g__Fen-1342;s__*
bin.91	*d__Bacteria;p__Planctomycetota;c__UBA1135;o__UBA2386;f__UBA2386;g__UBA2386;s__*
bin.34	*d__Bacteria;p__Patescibacteria;c__Gracilibacteria;o__UBA1369;f__UBA1369;g__PALSA-1335;s__*
bin.124	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__A4b;g__;s__*
bin.142	*d__Bacteria;p__Gemmatimonadota;c__Gemmatimonadetes;o__Gemmatimonadales;f__Gemmatimonadaceae;g__SCN-70-22;s__*
bin.125	*d__Bacteria;p__Planctomycetota;c__UBA1135;o__UBA2386;f__UBA2386;g__;s__*
bin.25	*d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__Phycisphaerales;f__SM1A02;g__;s__*
bin.133	*d__Bacteria;p__;c__;o__;f__;g__;s__*
bin.90	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Rubrivivax;s__*
bin.93	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter_E;s__*
bin.177	*d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Opitutales;f__Opitutaceae;g__UBA6669;s__*
bin.134	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__JOSHI-001;s__*

bin.59	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Anaerolineales;f__envOPS12;g__OLB14;s__*
bin.130	*d__Bacteria;p__Verrucomicrobiota;c__Lentisphaeria;o__;f__;g__;s__*
bin.36	*d__Bacteria;p__Bdellovibrionota;c__Bdellovibrionia;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio;s__*
bin.185	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__GCA-2729495;f__GCA-2729495;g__;s__*
bin.145	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Hyphomicrobium;s__*
bin.101	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagaceae;g__;s__*
bin.97	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__Thauera;s__*
bin.156	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Anaerolineales;f__envOPS12;g__OLB14;s__*
bin.54	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Promineofilales;f__Promineofilaceae;g__Promineofilum;s__*
bin.50	*d__Bacteria;p__Verrucomicrobiota_A;c__Chlamydiia;o__Parachlamydiales;f__FEN-1388;g__;s__*
bin.152	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Rubrivivax;s__*
bin.35	*d__Bacteria;p__Myxococcota;c__UBA9160;o__SZUA-336;f__;g__;s__*
bin.74	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__UBA7966;f__UBA7966;g__;s__*
bin.14	*d__Bacteria;p__Patescibacteria;c__Paceibacteria;o__UBA9983_A;f__;g__;s__*
bin.65	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__UBA3071;f__CG2-30-64-16;g__;s__*
bin.135	*d__Bacteria;p__Patescibacteria;c__Dojkabacteria;o__B142;f__;g__;s__*
bin.115	*d__Bacteria;p__Acidobacteriota;c__Acidobacteriae;o__Bryobacterales;f__;g__;s__*
bin.31	*d__Bacteria;p__Bacteroidota;c__Ignavibacteria;o__Ignavibacteriales;f__Ignavibacteriaceae_A;g__UTCHB3;s__*
bin.205	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Comamonas_C;s__*
bin.102	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Azospirillales;f__Azospirillaceae;g__;s__*
bin.192	*d__Bacteria;p__Patescibacteria;c__ABY1;o__SG8-24;f__2-12-FULL-60-25;g__SZUA-46;s__*
bin.144	*d__Bacteria;p__Bacteroidota;c__Ignavibacteria;o__Ignavibacteriales;f__Ignavibacteriaceae;g__Ignavibacterium;s__*
bin.104	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Rubrivivax;s__*
bin.55	*d__Bacteria;p__Patescibacteria;c__ABY1;o__Veblenbacterales;f__;g__;s__*
bin.38	*d__Bacteria;p__Acidobacteriota;c__Blastocatellia;o__Pyrinomonadales;f__Pyrinomonadaceae;g__OLB17;s__*

bin.12	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__UTPRO2;s__***
bin.89	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Rubrivivax;s__***
bin.69	***d__Bacteria;p__Patescibacteria;c__ABY1;o__SG8-24;f__2-12-FULL-60-25;g__2-12-FULL-60-25;s__***
bin.165	***d__Bacteria;p__Chloroflexota;c__Dehalococcoidia;o__UBA2991;f__UBA2991;g__G233;s__***
bin.190	***d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA1406;f__GWC2-37-13;g__GWC2-37-13;s__***
bin.44	***d__Bacteria;p__Patescibacteria;c__Paceibacteria;o__UBA9983_A;f__UBA2163;g__UBA10103;s__***
bin.20	***d__Bacteria;p__Patescibacteria;c__Saccharimonadia;o__Saccharimonadales;f__UBA10212;g__;s__***
bin.169	***d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA1400;f__UBA12028;g__PSRQ01;s__***
bin.180	***d__Bacteria;p__Patescibacteria;c__Gracilibacteria;o__UBA1369;f__;g__;s__***
bin.47	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Ga0077536;f__Ga0077536;g__Ga0077536;s__***
bin.100	***d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__Chitinophagaceae;g__Palsa-955;s__***
bin.121	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Palsa-1005;g__PALSA-1003;s__***
bin.155	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__GCA-2729495;f__GCA-2729495;g__;s__***
bin.103	***d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA10105;f__;g__;s__***
bin.183	***d__Bacteria;p__Actinobacteriota;c__Thermoleophilia;o__20CM-4-69-9;f__;g__;s__***
bin.161	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__UBA2357;s__***
bin.184	***d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA1406;f__GWC2-37-13;g__GWC2-37-13;s__***
bin.17	***d__Bacteria;p__Planctomycetota;c__Phycisphaerae;o__Phycisphaerales;f__SM1A02;g__UBA2402;s__***
bin.29	***d__Bacteria;p__Bacteroidota;c__Ignavibacteria;o__Ignavibacteriales;f__Ignavibacteriaceae;g__;s__***
bin.136	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__;s__***
bin.178	***d__Bacteria;p__Patescibacteria;c__Doudnabacteria;o__UBA920;f__UBA920;g__PALSA-1336;s__***
bin.109	***d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales_B;f__Magnetospirillaceae;g__;s__***
bin.61	***d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__UBA10324;g__;s__***
bin.42	***d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA1400;f__;g__;s__***
bin.16	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__;f__;g__;s__***

bin.194	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__A4b;g__;s__*
bin.49	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__Zoogloea;s__*
bin.106	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__UTPRO2;s__*
bin.8	*d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA1400;f__PJMF01;g__1-14-0-10-56-10;s__*
bin.7	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__Zoogloea;s__*
bin.78	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__koll-22;g__;s__*
bin.107	*d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales;f__UBA10324;g__;s__*
bin.204	*d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA1400;f__PJMF01;g__;s__*
bin.86	*d__Archaea;p__Nanoarchaeota;c__Nanoarchaeia;o__Woesearchaeales;f__ARS49;g__;s__*
bin.10	*d__Bacteria;p__Patescibacteria;c__Microgenomatia;o__UBA10105;f__UBA927;g__UBA927;s__*
bin.197	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Thermoflexales;f__;g__;s__*
bin.170	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__4572-78;f__;g__;s__*
bin.24	*d__Bacteria;p__Armatimonadota;c__UBA10988;o__UBA10988;f__;g__;s__*
bin.209	*d__Archaea;p__Nanoarchaeota;c__Nanoarchaeia;o__Woesearchaeales;f__ARS49;g__;s__*
bin.105	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__UBA2357;s__*
bin.154	*d__Bacteria;p__Chloroflexota;c__Anaerolineae;o__Anaerolineales;f__envOPS12;g__OLB14;s__*
bin.4	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__GCA-2729495;f__GCA-2729495;g__;s__*
bin.146	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Parvibaculales;f__Parvibaculaceae;g__;s__*
bin.22	*d__Bacteria;p__Bacteroidota;c__Ignavibacteria;o__Ignavibacteriales;f__Ignavibacteriaceae_A;g__;s__*
bin.200	*d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Opitutales;f__Opitutaceae;g__;s__*
bin.48	*d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__UTPRO2;s__UTPRO2 sp003105015*
bin.195	*d__Bacteria;p__Planctomycetota;c__SZUA-567;o__;f__;g__;s__*
bin.79	*d__Bacteria;p__Patescibacteria;c__Paceibacteria;o__UBA9983_A;f__Zambryskibacteraceae;g__;s__*
bin.95	*d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingopyxis;s__*
bin.11	*d__Bacteria;p__Bacteroidota;c__Ignavibacteria;o__Ignavibacteriales;f__Ignavibacteriaceae;g__Ignavibacterium;s__*

bin.2	***d__Bacteria;p__Planctomycetota;c__UBA1135;o__UBA2386;f__UBA2386;g__;s__***
bin.159	***d__Bacteria;p__Zixibacteria;c__;o__;f__;g__;s__***
bin.172	***d__Bacteria;p__Acidobacteriota;c__Blastocatellia;o__Pyrinomonadales;f__Pyrinomonadaceae;g__OLB17;s__***
bin.30	***d__Bacteria;p__Chloroflexota_A;c__Ellin6529;o__CSP1-4;f__CSP1-4;g__Palsa-1032;s__***
bin.208	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__UTPRO2;s__***
bin.201	***d__Bacteria;p__Armatimonadota;c__Fimbriimonadia;o__Fimbriimonadales;f__Fimbriimonadaceae;g__UBA2387;s__***
bin.174	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__Thauera;s__***
bin.202	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Rubrivivax;s__***
bin.153	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Comamonas_C;s__***
bin.198	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__;s__***
bin.32	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__;s__***
bin.164	***d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Rhodocyclaceae;g__;s__***

VITA

Trevar Walker Lytle

Candidate for the Degree of

Master of Science

Thesis: MOLECULAR METHODS TO DETECT REGULATORY GENES IN ANAEROBIC AMMONIA OXIDIZING BACTERIA

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